Banana Pests and Diseases

Field Guide for Disease Diagnostics and Data Collection

Improvement of banana for smallholder farmers in the Great Lakes Region of Africa

Improvement of banana for smallholder farmers in the Great Lakes Region of Africa

This unique project seeks to improve the production and productivity of banana in Tanzania and Uganda, through the development of hybrid banana varieties that are expected to have 30% higher yield compared to the current varieties grown by farmers under the same conditions through improved resistance against key pests and diseases. In particular, the project will strengthen the banana breeding programs in the two countries, towards developing new high-yielding and disease-resistant hybrid banana varieties, primarily focusing on the two most popular cooking bananas in the region: East Africa highland banana (EAHB), also known as Matooke, and on Mchare.

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Author information

A field guide on four important pests and diseases of banana was produced within the framework of the Bill and Melinda Gates Foundation-funded project on 'Improvement of banana for smallholder farmers in the Great Lakes Region of Africa'. The authors of the guide are scientists from four of the partners in the project: Stellenbosch University (SU), the International Institute for Tropical Agriculture (IITA), the National Agricultural Research Organization (NARO) in Uganda, and the Horticultural Research Institute (ARI) in Tanzania.

Stellenbosch University (SU) is amongst South Africa’s leading tertiary institutions based on research output, student pass rates and rated scientists, and is recognised internationally as an academic institution of excellence. The university is home to an academic community of 29 000 students (including 4 000 foreign students from 100 countries) as well as 3 000 permanent staff members (including 1 000 academics) on five campuses. As research partner, SU participates in various international academic networks. The scenic beauty of the Stellenbosch area; state-of-the-art, environmentally friendly facilities and technology; and the visionary thinking about the creation of a sustainable 21st-century institution, makes for the unique character of Stellenbosch University.

International Institute for Tropical Agriculture (IITA) is an Africa-based international not-for-profit research-for-development organization, established in 1967, and governed by a board of trustees. IITA is a member of the CGIAR Consortium, a global research partnership for a food secure future. IITA’s mission is to offer a leading research partnership that facilitates agricultural solutions for hunger, poverty, and natural resource degradation throughout the tropics. IITA has over 100 internationally recruited staff and around 1000 support staff based in various IITA stations across Africa. IITA’s mission is in line with that of the new CGIAR and focuses on the four system-level outcomes (SLOs): (1) increase in food security, (2) reduction of rural poverty, (3) reduction of undernutrition, and (4) more sustainable management of natural resources.
**National Agricultural Research Organization (NARO)** is the apex body for guidance and coordination of all agricultural research activities in the national agricultural research system in Uganda. NARO is a Public Institution established by an act of Parliament, which was enacted on 21st November 2005. NARO comprises of 15 semi-autonomous public agricultural research institutes across the various agro-ecological zones of Uganda. The Research capacity and reputations of the Public Agricultural Research institutes has been built over several decades, since 1898. For instance, banana research at NARO has been on for more than 25 years, generating and delivering evidence based technologies, management practices and policy options for improved nutrition, resilience and productivity in East and Central Africa. NARO has over time built its capacity (infrastructural and human), and dependable and formidable regional and international network of partners for delivering research and scaling outputs to the banana farming and trading communities.

**Horticultural Research and Training Institute (ARI-HORTI), Tengeru** was established during 1980 with the national mandate to undertake horticultural research and training activities, has main objective of supporting horticultural activities connected with training, research and extension on vegetable seed production and plant propagation. Research work at HORTI Tengeru is currently being conducted on vegetables (exotic and indigenous), fruits (exotic and indigenous), bananas, root and tuber crops, mushroom (oyster mushrooms) spices supported with unit responsible for liaising and dissemination of technologies to clients, with 40 resource personnel manning the institute. Among the current activities being carried out is: collection and conservation of local banana germplasm; expand the varietal choices and gene base of banana growers by exchanging and introducing selected superior banana varieties with enhanced pest and disease resistance and high yield potential from reputable breeding programs in the world; strength the capacity (institutional and human) for the transfer and application of improved banana production strategies by establishing links with partners (public and private); dissemination of improved husbandry practices; popularization of the use of clean planting materials; and management of pests and diseases (nematodes, weevils and soil fertility improvement, Fusarium wilt).
Introduction

Banana is a staple food in East and Central Africa (ECA), and provides approximately 20% of the total calorie consumed per capita. Uganda and Tanzania are two of the main producers of bananas in the region, mainly through the cultivation of two unique cooking types, the Matoke and Mchare bananas. Production of bananas in ECA, however, has declined since the 1970s, and now yields a fraction of its potential (Van Asten et al., 2005). While low yields are partly due to poor soil fertility in the region (World Bank, 2010), pests and diseases have played a significant role in reducing banana yields (Swennen et al., 2013).

The project Improvement of banana for smallholder farmers in the Great Lakes Region of Africa focuses on producing high-yielding banana hybrids by developing host plant resistance to diseases and pests important to the region. These diseases and pests include Fusarium oxysporum f. sp. cubense (Foc) which causes Fusarium wilt, banana weevils and nematodes, and Mycosphaerella spp. which cause Sigatoka leaf spots (Edmeades et al., 2007; Swennen et al., 2013). Pests and diseases pose a substantial problem to sustainable banana production in ECA, with a significant risk to destabilize food security and household income in this region.

Breeding for host plant resistance is the most appropriate method to control pests and diseases, as pesticides are often unaffordable, not available, dangerous to use or simply not an alternative (as is the case for Fusarium wilt). Laudable progress has been made in breeding and selection for combined resistance to Foc and nematodes (Rowe and Rosales, 2000), even though they do not meet the cooking-banana end-user preference for flavour, taste and texture. The National Agricultural Research Organization (NARO) of Uganda, in collaboration with the International Institute for Tropical Agriculture (IITA) has therefore developed 27 EAHB (NARITA) hybrids, of which two were released as cultivars in 2010. At least 20 of the NARITA hybrids will be tested in this project for agronomic performance, pest and disease resistance and fruit quality, with the objective to identify hybrids with at least 30% higher yield that are 50% more resistant to at least three target pest and disease constraints.

Breeding for resistance in banana is complicated by a number of constraints, including pathogen and pest diversity. Hence, screening against the different key races and species is essential. Field screening, also, complicates the evaluation process as it is time-consuming, expensive and often subjected to uneven natural infection levels. Standardised protocols are thus needed to rapidly and accurately test banana breeding lines and hybrids for resistance to pathogens and pests under controlled. In addition, molecular technologies to support pathogen identification and resistance screening are required.
The *Field guide for disease diagnostics and data collection* of banana pests and diseases have been prepared to assist banana scientists, technical staff and extension officers to collect pathogens and pests from testing and breeding sites in Uganda and Tanzania. These will then be sent for identification and characterization to Stellenbosch University, IITA, NARO and ARI. This is to ensure that field evaluation of hybrids and banana breeding efforts are performed against all variants of banana pathogens and pests in ECA. Methods to evaluate NARITA hybrids and other breeding materials for resistance to the target pathogens and pests are also presented. To accelerate the breeding process, small plant screening methods are proposed for Fusarium wilt, *Mycosphaerella* spp., nematodes and weevils.

**References:**


Banana Fusarium wilt

Background

Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *cubense* (Foc), is a destructive soil-borne disease of bananas present in all banana-producing countries in sub-Saharan Africa. The disease was first discovered in Australia in 1876, but it became prominent when it almost destroyed the international banana export industry based on Gros Michel bananas in the 1900’s. Because of the ability of Foc to survive in infested soils for decades, Fusarium wilt is extremely difficult to manage. The disease in Central America was brought under control when Gros Michel bananas were replaced with Cavendish cultivars; a banana variety which are not affected by the strain in Central America (Foc race 1). Foc race 1 is, however, also found throughout Africa. Cooking bananas and plantains grown in Africa are able to resist Foc race 1. Since the 1990s, Cavendish bananas in tropical Asia became severely affected by a new strain of Foc, called Foc TR4. The ability of African bananas to resist Foc TR4 is largely unknown.

Foc consists of three races and 24 vegetative compatibility groups (VCGs). Nine VCGs of Foc are present in Africa (Blomme *et al*., 2013), of which VCGs 0124 and 0125 are the most widely distributed (Karangwa, 2015) in East and Central Africa (ECA). Fusarium wilt has been moved to new growing areas by the movement of infected planting materials (Stover, 1962). Their occurrence in Africa is also strongly related to the distribution of susceptible banana cultivars (Blomme *et al*., 2013). The disease is thus mostly observed in areas where susceptible sweet bananas are grown, but not in fields planted to the African cooking (Matooke) bananas. Foc TR4 is only found in northern Mozambique, where it was introduced in 2013. This strain is expected to spread across ECA, and might affect both dessert and cooking bananas on the continent.

Laudable progress has been made in breeding banana hybrids resistant to Foc (Rowe and Rosales, 2000). Foc-resistant hybrids developed by FHIA (a banana breeding program in Honduras), such as the FHIA-17 and FHIA-25 hybrids, are appreciated in many parts of Africa (Dzomeku *et al*., 2008; Gaidashova *et al*., 2008; Karamura *et al*., 1999; Msogoya *et al*., 2006). Still, they do not meet the Matooke end-user preference for flavour, taste and texture. It is thus important that banana hybrids popular to African consumers be developed by introducing...
resistance from wild diploids into local varieties. To ensure that breeding efforts target all races and VCGs of Foc in ECA, rapid diagnostic methodologies have to be developed to accurately detect them. Reliable small plant screening methods are thus being developed to accelerate the banana breeding process.

Symptoms

Banana plants with Fusarium wilt can be identified by the conspicuous yellowing and wilting of older leaves which progress to the youngest leaves until affected plants are eventually killed (Fig. 1A). The disease should, however, not be identified only on these external symptoms, as several other biotic (bacterial wilt, Armillaria, banana weevil) and abiotic stresses (nutrient deficiency, water-logging) of banana can result in the yellowing and wilting of banana plants. For this reason, Fusarium wilt in suspect plants needs to be identified by also inspecting internal symptoms in the pseudostem and rhizome, and by isolating Foc from affected tissue.

External symptoms

The most characteristic symptom of banana Fusarium wilt is chlorosis (yellowing) of older banana leaves that progress upward. The yellowing most often start on the leaf margins, from where they progress to the leaf midriff (Fig. 1B). In some banana varieties, such as Cavendish bananas affected by Foc race 4, the chlorosis is followed by necrosis (browning caused by death of leaf tissue). In others, such as Pisang Awak, the browning of affected leaves are seldom observed. In Gros Michel affected by Foc race 1, yellowing might not be noticed on affected leaves (Fig. 2). Despite the variety involved, two other indicators are always associated with leaves affected by Fusarium wilt: the symptoms progress upwards to the younger leaves, and the petioles buckle and hang down the pseudostem.

A second external symptom often linked to banana Fusarium wilt is the splitting of the pseudostem (Fig. 1C). The splitting is caused by the inability of dead leaf bases to expand as the plant grows, thus splitting open as the inner pseudostem swells. Pseudostem splitting is not always associated with Fusarium wilt, and there might be other causes for the splitting of pseudostems too. This symptom, therefore, has to be considered along with leaf yellowing and wilting in suspect plants.
Internal symptoms

Fusarium wilt results in very characteristic internal symptoms in the rhizome and pseudostem, irrespective of the cultivar affected. When affected pseudostems are cut through horizontally, reddish- to dark-brown lesions can be spotted inside the leaf bases that form the pseudostem (Fig. 1E). The bunch stalk, however, will always be clean of such symptoms. It is always advisable to split the pseudostems longitudinally through the discoloured lesions, as their progression through the vascular tissue then becomes very clear. The youngest infection in banana pseudostems are often yellow to dark red, and limited to the xylem vessels only. This is the best material to collect for the isolation of Foc. The older the infection becomes, the more expanded and darker the lesions will become, and such lesions might be co-colonised by secondary contaminants. Despite the age of the lesion, it is important to confirm that they are continuous, which separates internal symptoms caused by Foc from those caused by other biotic stresses.

When external symptoms are visible on banana plants, but internal symptoms are absent from the pseudostem, it becomes important to inspect the rhizome. The plant then has to be cut open at soil level to expose the pseudostem base, and then pushed over. Diseased plants will have a very characteristic yellow to dark-red discolouration of the inner rhizome, which usually starts at the edges and progresses inwards (Fig. 1D). Very often only part of the inner rhizome is affected, but as the disease progresses, it might affect the entire inner rhizome. The outer rhizome is never affected. The part of the rhizome that had been pushed over (not cut) will display yellow strands of the rhizome which are attached to the both the top and bottom half of the rhizome. When no discolouration is observed within the rhizome, the external symptoms were caused by something different than Foc. In such cases, the inner rhizome might display black spots instead of the continuous yellow to reddish-brown discolouration associated with Fusarium wilt.
Figure 1. Disease symptoms of Fusarium wilt of banana caused by *Fusarium oxysporum* f. sp. *cubense* (A, B). Affected plants wilt rapidly, older and then younger leaves become yellow and brown, and plants eventually die. In some cases, the base of pseudostems splits (C). Internally, the vascular bundles in the pseudostem will turn yellow to reddish-brown (D), while a deep golden discolouration of the inner rhizome develops (E).
Figure 2. Disease symptoms of Fusarium wilt on different banana varieties: (A) Cavendish bananas in South Africa, (B) Pisang Awak in Uganda, (C) Pisang Awak in Mozambique, and (D) Gros Michel in Costa Rica.
Sample collection

Taking a sample from the diseased host plant

Samples for identification of the cause of Fusarium wilt should consist of a section from the pseudostem of the wilted banana plant where continuous discoloured vascular strands are evident (Fig. 3). The sample should be taken from as low in the pseudostem as possible, but not from areas where decay is advanced. Also, the sample should be taken from as close to the centre of the pseudostem as possible, as opposed to the outermost leaf bases. As banana tissue is very wet, the risk of bacterial contamination of samples is high, particularly in warm weather and samples can deteriorate rapidly. The chance of recovering healthy cultures of Foc decreases as the sample deteriorates. Samples should be kept in heavy paper bags or wrapped in paper until the strands can be excised. Avoid plastic bags as this causes the samples to sweat and promotes growth of bacteria. Accurate notes must be taken for each sample where applicable, including:

- Sample number (one sample number per plant)
- Date
- The variety of the host plant, including local names (and uses if known)
- Genomic constitution of host if known (e.g. AA, AAB, ABB etc.)
- Age of plant/plantation
- Whether plants sampled are grown in a garden, commercial plantation, village or the wild
- Size of the diseased area where the plant was collected, with photos
- Location (e.g. name of province/state, how far in what direction from nearest town, name of road, name of property if sample is from a commercial plantation etc.) A map with sample numbers marked on it and GPS coordinates essential.
- Collectors names, and the required phytosanitary certificate/importation permit
- Other useful observations might include the source of the planting material, whether the plant is growing in water-logged soil, how many plants are affected, what other varieties are growing in the vicinity and are these diseased or healthy?

A small (5x5 cm) piece of rhizome tissue showing typical discoloured vascular strands may also be used as a sample, but this is not recommended if decay in the rhizome is
advanced. This piece of rhizome tissue should also be wrapped in paper or placed in a paper envelope to dry.

**Note:** When looking for wilt-affected plants, it is better to take samples from established plantings of bananas rather than recently planted young plants.

**Figure 3.** Vascular strands are collected from banana pseudostems with typical Fusarium wilt symptoms (A). This can be achieved in a non-destructive way (B) by slicing open part of the pseudostem on the side of yellow leaves, and by dissecting out discoloured xylem tissue (C).

*Dissecting discoloured vascular strands from sample*

Ideally, the discoloured vascular strands should be dissected from the sample on the same day that it is collected, or as soon as possible after collection. The use of sterile blotting papers is recommended and aseptic techniques should be applied to the dissection of strands. Samples should first be surface-sterilised by wiping with 70% alcohol or surgical spirits. Where several samples are being prepared, a fresh piece of blotting paper should be used for each sample, and scalpel blades should be flamed if possible or at least wiped with 70% alcohol between samples. The excised strands should then be placed between sterile blotting papers in a paper envelope to dry naturally. A few days are usually sufficient. Do not let the strands get too hot (e.g. in direct sunlight or in the boot of a car) as this may kill the fungus. Do not dry them in an oven! Fusarium wilt specimens do not need to be kept in the fridge – room temperature
is OK. They do not need to be wrapped in moist paper like leaf specimens – dry paper is best.

**Posting of samples**

If posting the strands for isolation and analysis, please post in a paper envelope as soon as the strands are dry enough, with sample numbers and details clearly written on or with each sample envelope. Please include a copy of the relevant quarantine import permit inside the package if this is required.

**Note:** If there is any possibility that samples have been mixed up and the details for some samples may be incorrect, discard the samples concerned.

**Pathogen isolation**

*Isolating the fungus from discoloured vascular strands*

Isolations can be attempted when the strands have dried. Small sections (3-6 mm long) of dry discoloured vascular strands are submerged into plates of ¼ strength potato dextrose agar (PDA) medium amended with an antibacterial agent (e.g. streptomycin @ 1.2 mL/240 mL PDA). If present, Fusarium growth will appear from the strands in 2-4 days. However, if the sample is badly contaminated with bacteria this may mask fungal growth. Let samples dry further if this occurs and increase the strength of the antibacterial amendment in the media. A high rate of recovery of *Fusarium* should be expected from correctly prepared samples. Single-spore (monoconidial) cultures should be prepared from an isolate from each specimen.

*Single-sporing of isolates*

Single-spore isolates of *F. oxysporum* are obtained by dilution plating (demonstrated below). For both methods, a small scrape of sporulating hyphae are collected from cultures grown on ¼-strength PDA plates, and dissolved in 10 ml sterile distilled water in test tubes. From the initial spore suspension, a series of dilutions can be prepared. One ml of each of the dilution series is then pipetted onto water agar, and the water agar plates incubated with the lid up overnight at 25°C. The plates are viewed for germination of conidia under a dissecting microscope the following morning, and single-conidia cut from the water agar with a surface-sterilised scalpel and transferred to new 90-mm ¼-strength PDA plates. Additionally, single-spore cultures can also be
obtained by dissecting the very tip of single growing hyphae from an older culture grown on CLA.

**Maintenance of healthy cultures**

Healthy cultures of Foc from single spores are maintained on carnation leaf agar (CLA). Cultures can be initiated on weak-strength PDA medium (e.g. ½ strength) (Ainsworth, 1971) to check the morphology of cultures for taxonomic purposes or for spore production. Healthy (sporodochial-type) cultures of Foc growing on PDA medium exhibit abundant fluffy aerial mycelium after 2 days and produce abundant microconidia. Some macroconidia may also be produced on PDA although this type of spore is more commonly produced on CLA medium. Cultures of Foc should NOT be kept on PDA medium for longer than 4 or 5 days as mutations can rapidly occur and these cannot be reversed (Nelson *et al*., 1983, Windells, 1992). Mutated cultures (e.g. slimy pionnotal mutants) should be discarded. Cultures are normally maintained in an incubator at 25°C. Black light is generally not required for cultures of Foc to sporulate. Various methods are used for long-term (e.g. lyophilisation), medium-term (e.g. colonised filter paper in cold storage) and short-term (e.g. CLA) storage of cultures of Foc.
1. **Isolation from plant material.**

   Samples received

   ![Image of sample collection]

2. **Sub-culture small areas of good Fusarium growth onto Streptomycin PDA. Use these cultures for single-sporing (need to grow for 2-3 days to be sure of healthy culture).**

   ![Image of Streptomycin PDA]

3. **Place a spore suspension onto water agar.**

   ![Image of water agar]

4. **After 24 hours take at least 2 single germinated spores and place back onto Streptomycin-PDA.**

   ![Image of Streptomycin-PDA with small cube of agar]

5. **Once single spore cultures show normal Fusarium growth (should be visible after 4-5 days), choose only 1 single spore culture to represent each isolate and discard all other cultures. Assign this culture with a unique accession number. Subculture this isolate onto CLA, which will become the source for morphological, molecular and VCG identification, pathogenicity testing and storage.**

   ![Diagram of isolation process]

   **Figure 4.** Isolation of *Fusarium oxysporum* f. sp. *cubense* from diseased banana tissue.
Pathogen identification

Steps involved in laboratory diagnosis of Fusarium wilt of banana

- Receive specimen, log details and observations from grower/inspector.
- Isolate from symptomatic tissue (usually 2x Streptomycin-amended PDA plates with 4 pieces per plate).
- Check morphology of resultant growth (macro and microscopically).
- Subculture *Fusarium* colony to make spore suspension and plate onto water agar (WA).
- Select 2x germinated single spores to initiate monoconidial cultures.
- Assign unique accession number to isolate and record in specimen book and *Fusarium* isolate database.
- The mono-conidial culture is used to:
  a) Inoculate 2x PDA plates for cultural identification
  b) Initiate 2x carnation leaf agar (CLA) plates for morphological identification
  c) Inoculate 3x chlorate medium (CM) plates to generate *nit* mutants for VCG tests
  d) Inoculate 1x PD plate for DNA analysis
  e) Initiate 1x PDA plate for long-erm storage
  f) If necessary, prepare CLA slants for medium-term storage
  g) If necessary, arrange for lyophilisation of isolate for long-term storage in collection
- Conduct and record results of volatile and VCG analysis in database
- Return written reply to grower/inspector concerned using a Plant Disease Report form, recording the date and details of reply in specimen book (usually phone results also)
- Maintain isolate collections and records in database

Characteristics of *F. oxysporum* f. sp. cubense

- Produce abundant microconidia that are single-celled, oval to kidney-shaped
- Microconidia are produced in false heads on branched and unbranched monophialides
- Macroconidia are sickle-shaped with an attenuated apical cell and foot-shaped basal cell
• Chlamydosposes are present and formed singly or in pairs
• No perfect stage of *F. oxysporum* is known
• On PDA, fungal colonies produce white aerial mycelia that may turn purple in the centre. Isolates may differ in their cultural morphology
• Cream to orange sporodochia are formed on carnation leaves on CLA
Field and greenhouse trials

*Field evaluation of banana varieties for Fusarium wilt resistance*

**Preparation of plots**
It is important to consider banana production and disease management practices when selecting a location for field trials. The site should preferentially be located in a farmer's field, and managed according to existing production practices. Fertilisers and irrigation need to be applied according to existing procedures. Particular care should be taken not to allow the trial site so serve as a source of contamination to bordering plantations. De-suckering and leaf removal should continue in the trial site as usual, but fungicides should not be applied for the duration of the trial. The experiment should run for the plant crop and at least the first ratoon.

A proper knowledge of the history of a banana plantation is required when selecting trial sites for the evaluation of banana varieties for Fusarium wilt resistance. It is best to select an area where the disease is known to be well established and severe, and where a single Foc race is present. The inoculum in the soil should also be distributed as equally as possible. To achieve this, plants affected by Fusarium wilt will need to be chopped up and ploughed back into the soil. To increase inoculum in fields, susceptible banana varieties can be grown in future trials sites for one cycle before being chopped up and ploughed in. Alternatively, inoculum can be prepared in the laboratory and used to inoculate plants used in the trial. Inoculation of field sites should be done with extreme care, and only if necessary, without posing unnecessary risks to neighbouring fields. After the preparation of the field site, care should be taken to properly disinfect equipment used for the preparation of the soil, and also not to walk through the trial site unnecessarily during the execution of the trial. It is preferential that a specific team be appointed to maintain the field site and participate in the trial.

**Selection of varieties and controls**
When selecting varieties to be tested, it is important to target a specific Foc race and include control plants resistant and susceptible to that race (Table 1). It is also important to confirm the presence of the particular race in the trial site by characterising strains of Foc collected from diseased banana plants in that particular site. Three races
of Foc are known, each one specific to a single or a range of banana cultivars (Table 1). For instance, when varieties are screened for resistance to Foc race 1, Bluggoe/Gros Michel can be used as susceptible control and Cavendish as resistant control. If screened for resistance to Foc race 4, Silk/Cavendish can be used as susceptible control, and Calcutta-4 as the resistant control.
All plants used for field trials should be multiplied *in vitro* to prevent contamination with Foc (asymptomatic infections) and other pests and diseases of banana. If possible, they should also be virus-indexed. The plants should then be hardened-off under shade cloth in a nursery before field planting, either at the tissue culture facility where they were produced or at the farm where they will be planted. Special care should be taken not to contaminate the plants with Foc during the hardening-off stage by using Foc-free potting soil and irrigation water. Once plants reach a height of between 0.3 and 0.5 m, they can be taken to the field for planting. In the field, Fusarium wilt is expected to develop 3-12 months after planting, depending on the susceptibility of varieties and inoculum load in the soils. After disease development, pseudostem samples should be collected for the isolation and characterisation of Foc strains.

### Experimental design

Field experiments with Foc should be conducted by using a randomised complete block design (RCBD). A RCBD is used when a source of variation, such as the unequal distribution of Foc in banana soils, is present at the experimental site. By planting several plants from the same variety (treatments) in blocks that are randomised in the field, the experimental error caused by the unequal distribution of Foc in soils is reduced or eliminated. The effect of Foc on the treatments (banana varieties) are thus held constant, while the ability of the varieties to resist infection is allowed to vary.

Blocks usually consist of between 10 and 50 plants, with 3-10 replications of each block. Control plants should be included in the trial plan as a treatment. The number of plants/block and the number of replications/treatment will depend on the size of the field site and the cost of the trial. The greater the number of plants and blocks, the more accurate the outcome of the experiment will be. The level of infection

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**Table 1.** Banana varieties used as resistant and susceptible checks for evaluation against races of *Fusarium oxysporum* f. sp. *cubense*.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Susceptible banana varieties</th>
<th>Resistant banana varieties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foc race 1</td>
<td>Silk, Gros Michel</td>
<td>Cavendish, Bluggoe, Calcutta-4</td>
</tr>
<tr>
<td>Foc race 2</td>
<td>Silk, Bluggoe, Gros Michel</td>
<td>Cavendish, Calcutta-4</td>
</tr>
<tr>
<td>Foc race 4</td>
<td>Silk, Cavendish, Gros Michel, Bluggoe</td>
<td>Calcutta-4</td>
</tr>
</tbody>
</table>
of Foc in soils can also be determined by planting susceptible plants as a border row around each block, or even next to each of the plants tested in the trial.

**Data collection**

Data for disease progression will be collected monthly after 3 months. Field evaluation of trials will be based on external leaf symptoms *only*. The reason why other external symptoms are not considered is because pseudostem splitting is not a reliable indicator of disease severity, while rhizome discolouration requires destructive evaluation and can be performed only after harvest or at the end of the trial. Either way, it does not provide a reliable reflection of either disease incidence or disease severity.

Disease development can be measured on a 1-5 scale (Table 2). In some cases early symptoms of Fusarium wilt (Level 2) can be incorrectly identified. In such cases, the disease will not progress to Level 3 and beyond. In other cases, the disease could be correctly identified, but the plants will respond and the disease will disappear as new leaves emerge and older leaves are trimmed away. In most cases, however, the disease will progress beyond Level 2. Once it reaches Level 4 the plant will eventually die. It is thus important to not only rate the level of disease, but also the rate at which the disease develops. Since disease development depends on soil and environmental factors, an average rating per block will be determined, and the treatments compared by statistical analysis of a replicated trial.

**Table 2.** Disease rating scale for the evaluation of external symptoms of banana Fusarium wilt.

<table>
<thead>
<tr>
<th>Disease rating scale</th>
<th>Symptoms observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No visual leaf symptoms</td>
</tr>
<tr>
<td>2</td>
<td>0-33% of older banana leaves turning yellow</td>
</tr>
<tr>
<td>3</td>
<td>34-66% of older leaves turning yellow, with some hanging down the pseudostem</td>
</tr>
<tr>
<td>4</td>
<td>76-100% of leaves turning yellow and necrotic, with leaves hanging down the pseudostem</td>
</tr>
<tr>
<td>5</td>
<td>Plant dead, with brown leaves hanging down pseudostem</td>
</tr>
</tbody>
</table>
Susceptibility of banana varieties to Foc can be measured in two ways: by determining disease incidence and by determining disease severity. In addition, resistance can be measured by considering disease progression over time. The criteria that need to be fulfilled when measuring the effect of Fusarium wilt on bananas in field sites include the reliability, practicality, time and cost efficiency of the experiment, and data collection.

Disease incidence, severity and progression are calculated as follows:

**Disease incidence** indicates the proportion or percentage of plants diseased within a sampling unit (Seem, 1984). It is calculated by dividing the number of Fusarium wilt-affected plants by the total number of plants at a specific time point.

**Disease severity** quantifies the area of plant tissue affected. It may also be assessed by assigning a disease severity category or class value to each observed plant. It is calculated according to Sherwood and Hagedorn (1958) as:

\[
\text{Disease severity} \text{ (\%)} = \frac{\sum [(\text{number of plants in disease scale category}) \times (\text{specific disease scale category})]}{(\text{total number of plants}) \times (\text{maximum disease scale category})} \times 100
\]

**Disease progression** indicates the increase in disease incidence and/or severity over time, and can be measured by comparing disease progress curves of the different treatments.

Additional information can also be collected during disease ratings of Fusarium wilt. These include the time from planting to death of plants and yield. Field trials can further be used to test management options such as biological, chemical and cultural control. It is not recommended to use multiple fields for the same experiment, as the initial inoculum in fields may differ.

*Greenhouse evaluation of banana varieties for Fusarium wilt resistance*

Different greenhouse methodologies have been developed and are used by a number of researchers around the world. Greenhouse trials, however, should not be considered a replacement for field trials when assessing disease management options. In fact, results obtained in the greenhouse is often in conflict with those
obtained in the field. It is thus important to select an appropriate greenhouse test to provide preliminary data before experiments are repeated in the field. Two issues should be considered:

1. Greenhouse testing for disease resistance can be misleading, as disease development might disqualify entries from further use even though they can resist Fusarium wilt in the field. A good example is that Cavendish bananas often develop disease symptoms when inoculated with Foc race 1 in the greenhouse, but not in the field.

2. It is important to know what greenhouse method to use when performing Fusarium wilt trials, as inoculum load, soil type, plant age and greenhouse conditions all can affect the outcome of experiments. Preferentially, pilot trials should first be conducted to optimise experimental conditions when first performing Fusarium wilt tests in a new greenhouse. A selection of greenhouse tests, and their applications, are provided in Table 3.
Table 3. Greenhouse tests for banana Fusarium wilt and *Fusarium oxysporum* f. sp. *cubense*

<table>
<thead>
<tr>
<th>Trait tested for</th>
<th>Test used</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease resistance</td>
<td>In <em>vitro</em> bioassay</td>
<td>Wu <em>et al</em>., 2010</td>
</tr>
<tr>
<td></td>
<td>Root dipping</td>
<td>Mohammed <em>et al</em>., 2000; Ribeiro <em>et al</em>., 2011</td>
</tr>
<tr>
<td></td>
<td>Root dipping + millet seed</td>
<td>Dita <em>et al</em>., 2011</td>
</tr>
<tr>
<td></td>
<td>Soil drenching</td>
<td>Smith <em>et al</em>., 2008</td>
</tr>
<tr>
<td></td>
<td>Millet seed inoculation</td>
<td>Smith <em>et al</em>., 2008</td>
</tr>
<tr>
<td></td>
<td>Biochemical and structural variations in infected plants</td>
<td>Morpurgo <em>et al</em>., 1994; De Ascensao and Dubery, 2000</td>
</tr>
<tr>
<td>Biological/chemical control</td>
<td>Fungicides and sterilants</td>
<td>Nel <em>et al</em>., 2007</td>
</tr>
<tr>
<td></td>
<td>Biocontrol agents</td>
<td>Saravanan <em>et al</em>., 2003</td>
</tr>
<tr>
<td>Pathogen vs non-pathogen</td>
<td>Root dipping/ Immersion</td>
<td>Sun and Su, 1984; Ribeiro <em>et al</em>., 2011</td>
</tr>
<tr>
<td></td>
<td>Hydroponic cup test</td>
<td>Groenewald <em>et al</em>., 2006</td>
</tr>
<tr>
<td></td>
<td>Root dipping + millet seed</td>
<td>Dita <em>et al</em>., 2011</td>
</tr>
<tr>
<td>Race identification</td>
<td>Susceptible cultivars</td>
<td>Stover and Ploetz, 1990; Ploetz, 2000</td>
</tr>
<tr>
<td></td>
<td>VCG analysis</td>
<td>Ploetz, 2006</td>
</tr>
<tr>
<td></td>
<td>Molecular markers</td>
<td>Lin <em>et al</em>., 2009; Dita <em>et al</em>., 2010</td>
</tr>
</tbody>
</table>

**Preparation of trials**

Plants selected for greenhouse trials should preferentially be multiplied in a tissue culture facility. Depending on the nature of the trial conducted, the plantlets should then be grown in seedling trays, planting bags or pots to an appropriate size. For testing for resistance to Foc races, trials should preferentially be grown in potting soil to at least 30 cm before inoculation.

The Foc strain used for pathogenicity testing should have been single-spored and well-characterised before inoculation. It should then be plated onto ½ strength PDA and transferred to an appropriate medium to prepare inoculum. These media could be:

1. **Spore suspension**: Spore suspensions are used to either dip seedlings in before planting to pots, or for draining soils after plants had been planted. The suspensions are prepared...
by transferring Foc into Amstrong media (Booth, 1971), or by collecting conidia from PDA plates and suspending these into sterile distilled water. The concentration of spores is usually adjusted to $1 \times 10^6$ spores/ml for pathogenicity testing with Foc.

2. **Solid medium:** PDA pellets with Foc can be grown on sterile millet seeds or on a bran:sand mixture. These can then be mixed into the potting soil before the banana plants are transplanted into such soils, or added to the surface of the soil after transplanting.

### Rating of external symptoms on Cavendish/Mchare bananas in the field

<table>
<thead>
<tr>
<th>Disease rating scale</th>
<th>Symptoms observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No visual leaf symptoms</td>
</tr>
<tr>
<td>2</td>
<td>0-33% of older banana leaves turning yellow</td>
</tr>
<tr>
<td>3</td>
<td>33-66% of older leaves turning yellow, with some hanging down the pseudostem</td>
</tr>
<tr>
<td>4</td>
<td>66-100% of leaves turning yellow and necrotic, with leaves hanging down the pseudostem</td>
</tr>
<tr>
<td>5</td>
<td>Plant dead, with brown leaves hanging down pseudostem</td>
</tr>
</tbody>
</table>

![Rating: 1](rating1.jpg)  ![Rating: 2](rating2.jpg)  ![Rating: 3](rating3.jpg)
### Rating of external symptoms on Sukari Ndizi/Pisang Awak in the field

<table>
<thead>
<tr>
<th>Disease rating scale</th>
<th>Symptoms observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No visual leaf symptoms</td>
</tr>
<tr>
<td>2</td>
<td>0-33% of older banana leaves turning yellow</td>
</tr>
<tr>
<td>3</td>
<td>33-66% of older leaves turning yellow, with some hanging down the pseudostem</td>
</tr>
<tr>
<td>4</td>
<td>66-100% of leaves turning yellow and necrotic, with leaves hanging down the pseudostem</td>
</tr>
<tr>
<td>5</td>
<td>Plant dead, with brown leaves hanging down pseudostem</td>
</tr>
</tbody>
</table>

**Rating:**
- Rating: 1
- Rating: 2
- Rating: 3
- Rating: 4
- Rating: 5
Rating of external symptoms in the greenhouse

**Rating: 1**
Symptoms: No yellowing of leaves

**Rating: 2**
Symptoms: Yellowing of < 1/3 of the leaves

**Rating: 3**
Symptoms: Yellowing of 1/3 to 2/3 of leaves

**Rating: 4**
Symptoms: Yellowing of > 2/3 of leaves

**Rating: 5**
Symptoms: Plant dead
### Rating of internal symptoms in the greenhouse

<table>
<thead>
<tr>
<th>Disease rating scale</th>
<th>Symptoms observed</th>
<th>Disease rating scale</th>
<th>Symptoms observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No internal symptoms</td>
<td>4</td>
<td>1/3-2/3 Discoloured</td>
</tr>
<tr>
<td>2</td>
<td>Few internal spots</td>
<td>5</td>
<td>&gt;1/3 Discoloured</td>
</tr>
<tr>
<td>3</td>
<td>&lt;1/3 Discoloured</td>
<td>6</td>
<td>Entire inner rhizome</td>
</tr>
</tbody>
</table>

![Rating: 1](image1)

![Rating: 2](image2)

![Rating: 3](image3)

![Rating: 4](image4)

![Rating: 5](image5)

![Rating: ](image6)
References


Stover, R.H. 1962. Fusarial wilt (Panama disease) of bananas and other Musa species. Commonwealth Mycological Institute, Kew, Surrey, UK.


Karangwa, 2015


Appendix A

Inoculums preparation
1. *Fusarium oxysporum* f. sp. *cubense* is freshly isolated from susceptible plants on ½ strength potato dextrose agar (PDA) for 1 week, followed by conidia (macro- and microconidia) isolation and preparation in Armstrong's Liquid Medium. Cultures are incubated at room temperature and shaken twice a day for 7 days and then are filtered through two layers of cheesecloth. The desired inoculum concentrations of microconidia are prepared using a haemocytometer.

2. Alternatively; *Fusarium oxysporum* f. sp. *cubense* can be germinated on millet grains. *Foc* is freshly isolated from susceptible plants onto full strength PDA medium supplemented with 200 µg/mL ampicillin, and incubated for 7 days. Millet grain is washed, soaked and autoclaved to sterilise it. After cooling, uniformly growing fungus on PDA agar strips are cut using sterile blades and inoculated into sterile millet. The culture is incubated at room temperature and mixed daily for 10 days to ensure even distribution of the growing fungus. Conidia concentration is determined using a haemocytometer.

Inoculation procedures
1. **Inoculation by dipping:** Tissue culture generated plantlets are transferred to the double-pot system that is filled with sterilised sand or sand-soil mixture for hardening. They are then grown in the greenhouse until the desired size of about 15-25 cm high. After 45 days of acclimatisation the test plantlets are carefully uprooted, the roots washed with tap water, and plantlets with healthy white roots inoculated by immersion in a conidial suspension of $10^6$ conidia/mL for 2 hrs. They are then labelled and replanted in the trays for maintenance and observation in the greenhouse. Controls are immersed in water for 2 hrs. Leaf symptoms on susceptible plants are observed within 10-14 days of inoculation.

2. **Millet grain inoculation:** Well rooted and hardened tissue culture plantlets in the greenhouse are used. Double layered pots are half filled with sterile soil at the bottom of the pot and a known volume/weight of millet grain inoculums in the middle. Plants are uprooted with their soil in the previous pot, roots tips are wounded at the base and placed on the millet grain inoculums in the other pot. Plants are then watered regularly and are monitored for the development of the disease symptoms.

3. **Dispensing method:** A known volume of mycelial suspension (50 ml) is poured in the dug hole of about 3 cm at the base of the plant. Watering is delayed for 3 days, and disease symptoms observed after 2 weeks. Data on leaves showing symptoms is collected for a period of 2 months and rated according to the discolouration Rhizome Discoloration Index (RDI).
Yellow and Black Sigatoka

Background

Sigatoka leaf spot refers to a disease caused by number of ascomycete fungi in the genus *Mycosphaerella*. The three most important species associated with Sigatoka disease of banana are *M. fijiensis* (Stewart *et al*., 1999) that causes black leaf streak disease (black Sigatoka), *M. musicola* causing yellow Sigatoka (Stover and Simmonds, 1987), and *M. eumusae* causing eumusae banana leaf spot (Carlier *et al*., 2000b; Crous and Mourichon, 2002). *Mycosphaerella* species are among the most important constraints to banana production worldwide (Carlier *et al*., 1996). The pathogens damage banana leaves by causing necrotic leaf lesions that reduce the photosynthetic capacity of the plants, which result in reduced crop yield and fruit quality. This accounts for major economic losses and banana yield losses can be as much as 20-50% (Crous and Mourichon, 2002; Stover, 1983).

Of the three *Mycosphaerella* species causing Sigatoka leaf spot, *M. fijiensis* is considered the most damaging (Carlier *et al*., 2000). The disease affects banana leaves and results in severe necrosis, reducing the photosynthetic capacity of the plants and can result in yield losses of 50% or more. In Uganda, a 30-50% loss due to black Sigatoka had been reported (Mobambo *et al*., 1993), thereby posing a serious challenge to subsistence farmers. Other losses arise from premature fruit ripening, a serious defect in exported fruit (Stover, 1991). *Mycosphaerella fijiensis* has a short generation time, sporulates abundantly, and has a sexual cycle that enables the development of new resistance phenotypes hence it is classified as a high risk pathogen (Ploetz, 2000). Primary infections are usually generated from ascospores when conditions are conducive (e.g. high moisture) for disease development. Infected planting material and leaves, which often are used in developing countries as packaging materials, are usually responsible for long-distance spread of the disease (Ploetz and Mourichon, 1999).

*Mycosphaerella fijiensis* might have been present in Africa since 1973, but was first identified in Gabon in 1978 (Frossard, 1980). It is believed that the pathogen was introduced into Africa from infected banana plants imported from Asia. After this the disease has spread to Cameroon, Cote d’Ivoire, the Democratic Republic of Congo, Nigeria and Ghana. A separate introduction is believed to have brought the pathogen to the countries on the east coast of Africa (Carlier *et al*., 2000a). The disease was first identified in this region in 1987 on the island of Pemba. The disease quickly spread to the adjacent island of Zanzibar and from there to the mainland of Tanzania (Dabek and Waller, 1990). The disease has been reported in East African countries of Rwanda, Burundi, (Sebasigari and Stover, 1988), Uganda (Tushemereirwe and Waller, 1993), Kenya (Kung’U *et al*., 1992) and in Malawi (Ploetz *et al*., 1992).
Symptoms
Black Sigatoka and yellow Sigatoka can cause indistinguishable symptoms depending on the cultivar infected, stage of the disease and the season of the year (Johanson, 1993). However, yellow Sigatoka is characterised by oval to round necrotic lesions, which first appear pale yellow on the lower surface of the leaf (Meredith and Lawrence, 1970).

*Black Sigatoka*

The first symptoms appear as dark brown specks on the lower surface of the leaf. Stover and Simmonds (1987) described the black Sigatoka symptom progression as follows:

**Stage 1:** Faint, minute, reddish-brown specks on the lower surface of the leaf.

**Stage 2:** Specks elongate, becoming slightly wider to form narrow reddish-brown streaks.

**Stage 3:** Streaks change colour from reddish brown to dark brown or black, sometimes with a purplish tinge, clearly visible at the upper surface of the leaf.

**Stage 4:** The streaks broaden and become more or less fusiform or elliptical in outline and a water soaked border appears around each lesion.

**Stage 5:** The dark brown or black centre of each lesion becomes slightly depressed and water soaked border becomes more pronounced.

**Stage 6:** The centres of the lesions dry out becoming light grey or buff coloured and a bright yellow transitional zone appears between them and the normal green colour of the leaf. The lesions remain clearly visible after the leaf has collapsed or withered because of their light coloured centre and dark border (Table 4).

*Yellow Sigatoka*

Yellow Sigatoka can be differentiated from black Sigatoka at the early stages of lesion development (Stages 1 and 2) on visual symptoms. At later stages, examination of the conidiophores and conidia requires compound microscopy. There have been several descriptions of the development of individual lesions of Sigatoka disease over the years which were well summarised by Meredith (1970). Brun’s (1963) description is similar to that of Leach (1946), except that Brun excluded Leach’s 5th stage (second spot stage).
A brief description yellow Sigatoka symptom progression is found below:

**Stage 1:** This stage is characterised by the appearance of very small light green dots or dashes of approximately 1 mm in length.

**Stage 2:** The small dot or dash of Stage 1 elongates into a light green streak several millimetres long.

**Stage 3:** At this stage there is a change in the colour of the streak to a rusty brown. The streak becomes elongated and widens slightly. The border of the streak is ill defined.

**Stage 4:** The streak becomes more elliptical and is a definite spot with a sunken dark brown centre. It is often surrounded by a yellow halo. At this stage the conidia and conidiophores are produced.

**Stage 5:** The final stage has a grey dried out centre and an obvious black margin. This black margin can still be seen even after the leaf has dried out. (Table 3)

*Other Leaf spots*

A third leaf spot disease, Eumusae leaf spot, has recently been described. This disease is caused by the pathogen, *Mycosphaerella eumusae*, anamorph *Pseudocercospora eumusae* which is very closely related to *M. fijiensis* and *M. musicola* (Carlier et al., 2000; Crous and Mourichon, 2002). Distribution of this pathogen is still uncertain, but has been reported in South and South-east Asia. Little is known about *M. eumusae* but morphologically it is similar to *M. musicola*. It is also important to note that several other leaf spot diseases may produce lesions with a similar appearance to those of *M. fijiensis* and *M. musicola*, however these other pathogens can be easily distinguished using light microscopy as they are morphologically quite different.
### Table 4. Pictorial representation of Sigatoka disease symptoms.

<table>
<thead>
<tr>
<th>Stage 1</th>
<th>Black Sigatoka</th>
<th>Yellow Sigatoka</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image1" alt="Black Sigatoka" /></td>
<td><img src="image2" alt="Yellow Sigatoka" /></td>
</tr>
<tr>
<td>Stage 2</td>
<td><img src="image3" alt="Black Sigatoka" /></td>
<td><img src="image4" alt="Yellow Sigatoka" /></td>
</tr>
<tr>
<td>Stage 3</td>
<td><img src="image5" alt="Black Sigatoka" /></td>
<td><img src="image6" alt="Yellow Sigatoka" /></td>
</tr>
<tr>
<td>Stage 4</td>
<td><img src="image7" alt="Black Sigatoka" /></td>
<td><img src="image8" alt="Yellow Sigatoka" /></td>
</tr>
<tr>
<td>Stage 5</td>
<td><img src="image9" alt="Black Sigatoka" /></td>
<td><img src="image10" alt="Yellow Sigatoka" /></td>
</tr>
<tr>
<td>Stage 6</td>
<td><img src="image11" alt="Black Sigatoka" /></td>
<td><img src="image12" alt="Yellow Sigatoka" /></td>
</tr>
</tbody>
</table>
**Disease cycle and epidemiology**

The disease cycle for both *M. fijiensis* and *M. musicola* is similar with only minor differences as outlined previously. As *M. fijiensis* produces considerably less conidia and for a shorter period of time than *M. musicola*, ascospores are the main dispersal agent for this pathogen (Stover, 1980). Both conidia and ascospores are important for dispersal of *M. musicola* (Stover, 1971), however for both pathogens, ascospores are involved in the movement of the pathogen over longer distances rather than conidia. A distinctive line spotting pattern of infection is produced when the source of inoculum is conidia dislodged by rain splashes. These run down the inside of the cigar leaf cylinder contacting the lower point of the cylinder resulting in a line of infection. The deposition of ascospores by wind currents is generally on the terminal end of these leaves resulting in a distinctive leaf tip infection (Meredith, 1970).

The disease cycle is much faster for black Sigatoka than it is for yellow Sigatoka, as seen by the earlier appearance of spots. Inoculation studies conducted in Honduras demonstrated that spotting associated with *M. fijiensis* infections appeared 8-10 days faster than that associated with *M. musicola* infections. Ascospore maturation time is also shorter at 2 weeks for *M. fijiensis* compared with 4 weeks for *M. musicola* (Stover, 1980). A diagrammatic representation of the disease cycle for *M. fijiensis* is presented in Figure 5.
Sample collection

Sampling from the diseased host plant in the field

The sample should consist of a section from the leaf bearing numerous Sigatoka disease lesions at the stage 2 to 4 according to Brun’s scale (1963). Selected samples should also bear young active stages of the disease. For ascospore discharge, samples should be taken from necrotic parts of the leaves. Samples should be kept in heavy paper bags or wrapped in paper (e.g. newsprint) if the sampled fields are close to or will be transported to the laboratory quickly. Avoid using plastic bags as this increases humidity and promotes growth of bacteria and samples might rot. For collecting samples in fields far away from the laboratory, or if samples will not be taken to the laboratory immediately, use the method outlined in Fig. 7. Accurate notes must be taken for each sample including:

- Sample number (one sample number per plant)
- Date sampled
- The variety of the host plant, including local names (and uses if known)

Figure 5. Life cycle of black Sigatoka. Adapted from “Bennett, R.S. and P.A. Arneson. 2003. Black Sigatoka of bananas and plantains. The Plant Health Instructor. DOI:10.1094/PHI-I-2003-0905-01”.
• Whether the plants sampled are growing in a garden, commercial plantation, village or location (e.g. name of province or state, village, district, farmer’s name, etc.) marked on it or.
• GPS coordinates and altitude
• Collector’s names
• Other useful observations might include the source of the planting material, how many plants are affected what other varieties are growing around the diseased plant and are these diseased or healthy?
• Disease assessment parameters (see below)

**Materials**
Scissors, cardboard boxes; herbarium presser; news print; ropes for tying the samples together, paper bags (Fig. 6).
1. Sample collection: cut a piece of leaf with good clear symptoms and some green tissue. Avoid heavily necrotic regions. Sterilise equipment to avoid contamination.

2. Assemble Herbarium presser: put piece of cardboard on the presser, and news print, on which to tap the leaf sample.

3. Fix sample on news print using masking tape to firmly hold the sample in place; write necessary information: e.g. GPS coordinates, variety; farmer name, location, date, collector, etc.

4. Cover sample with news print, the piece of cardboard. This will separate samples and avoid contamination. The news print will absorb moisture; cardboard will press the sample for preservation. Many samples can be collected and put on a single presser.

5. When sampling is finished, tie samples together (tightly) so they are pressed. Take samples to the laboratory. Samples can be stored on the shelves for long periods without losing pathogenicity. It is important to remove moisture to avoid sample rotting.

**Figure 6.** Sampling banana leaves with Sigatoka symptoms for identification of *Mycosphaerella* spp.

**Pathogen isolation**

*Isolation and identification of Mycosphaerella spp.*

Several protocols / methods can be used to obtain pure isolates of *Mycosphaerella* species. The choice of the method / protocol depends on availability of materials required for isolation. Following purification, sporulation is induced and resulting fungal structures (conidiophores and conidia) are used for identification of *Mycosphaerella*.
spp. associated with observed leaf spots. Pure isolates can be obtained by (i) directly plating symptomatic leaf tissue on PDA media; (ii) picking conidia from sporulating lesions from the field; or (iii) from ascospores discharged onto media.

**Direct isolation**

To produce cultures directly from leaf material, early disease development stages are required (Figure 7). Material is surface sterilised and small pieces of tissue are excised and directly plated on PDA media.

![Figure 7](image)

**Figure 7.** Lesion stage required for direct isolation of *Mycosphaerella* spp. (*M. musicola* and *M. fijiensis/M. eumusae*) from symptomatic leaf tissue.

**Procedure**

1. Select leaf material containing Stage 2 lesions of suspected *M. musicola*, or Stage 2-3 for *M. fijiensis* or *M. eumusae* and cut into 1-2 cm squares
2. Immerse tissue into a beaker containing 1% sodium hypochlorite for 1 min, remove and wash five times with sterile distilled water, blot dry using sterile blotting paper
3. Using a sterile scalpel blade, make incisions on either side of the lesion, then horizontally, taking care not to cut right through the leaf piece
4. Excise epidermal pieces of tissue (approximately 2mm square) and plate onto PDA
5. Seal the plate with Parafilm® and incubate at 25°C for 10 days
6. Transfer small portions (hyphal tips) of the culture to V8 media to induce conidia production.

Mount conidia on a glass slide and observe under compound microscope to identify *Mycosphaerella* species using features described in Table 3.
Other protocols for isolating single conidia from banana leaves to start a pure culture

Method 1:
1. Surface sterilise leaves (cut in 4×4 cm squares) with either Fouré stages 2-5 with 20% commercial sodium hypochlorite bleach plus Tween 20 (500 μL/L) for 10 min.
2. Dry the sterilised leaves with sterile paper towels.
3. Place dried sterile leaves on top of sterile filter paper soaked with sterile distilled water.
4. Seal inside a Petri dish with wet filter paper.
5. Incubate plates at 20°C for up to 5 days to induce conidia production.
6. Using a small piece of water agar, light touch the top of the sporulating lesion and put the agar piece in an Eppendorf tube with sterile distilled water
7. Vortex for 2 minutes to dislodge the spores in the water and spread 200 μl of water on water agar and incubate for 8-12 h at 25 °C with continuous white light.
8. Under a stereo microscope, locate a germinating spore and transfer to a fresh plate to start a pure culture of Mycosphaerella spp., and incubate at 25ºC for 10 days.
9. Transfer small portions (hyphal tips) of the culture to V8 media to induce conidia production and use these for identification as described above.

Or,
1. Using pieces of the leaves directly from the field (if sporulation is occurring),
2. Press the abaxial surface of the infected leaves for M. fujensis and the top for M. musicola and M. eumusae (directly from the field) against a water-agar (3%) plate.
3. Observed under a stereoscopic microscope.
4. Using the needle of a subdermal syringe, pick single conidia and incubated on potato-dextrose-agar (PDA) plates containing 200 mg/mL Amoxicillin.
5. Grow single-spore isolates at 25°C with continuous white light at 25ºC for 10 days.
6. Transfer small portions (hyphal tips) of the culture to V8 media to induce conidia production and use these for identification as described above

1. Keep fresh leaf samples in envelopes and maintain at 5°C (2-3 days) until processing.
2. Make single conidial isolations from stage 5 leaf spots for respective sample.
3. Pipette 50 μl of sterile distilled water onto mature lesions and leave for 10 s and then transfer to 50 μl sterile distilled water in an Eppendorf tube.
4. Thoroughly mix the suspension and spread over the surface of 20 g/L water agar.
5. Incubate plates at 25°C for 1-2 days to induce conidial germination.
6. Transfer germinating conidia to half-strength PDA (PDA, Merck, 19 g/L PDA + 10 g/L agar) supplemented with 0.2 g/L Novobiocin.

**Production of cultures from ascospores**

Cultures of *Mycosphaerella* spp. can be derived from ascospores (sexual stage). These cultures can be used to produce conidia (asexual stage) for species identification (Fig. 8). Several methods are used; the choice depends on material availability and experience of the researcher. All work very well and achieve the desired objective. The ascospore method requires dried leaf tissue containing necrotic lesions which are wetted causing the perithecia to rupture and eject their ascospores (Fig. 8). Germinated ascospores are removed to PDA and subsequently subcultured onto sporulation medium to produce conidia for identification.

**Materials and methods**

- Scalpel blades
- 90mm diameter Petri dishes
- 90 mm diameter Whatman filter paper 1% and 2% sodium hypochlorite solution
- Sterile distilled water
- Sterile blotting paper
- Tweezers
- 25°C incubator with 12hr day/12hr night light cycle
- Dissecting microscope with light source underneath stage
- Bunsen burner
- Parafilm®
- 3% water agar plates
- PDA

**Procedure ascospore ejection**

1. Select leaf tissue with necrotic lesions (most likely to contain ascospores), particularly those with grey centres which contain small black spots (perithecia) (Fig. 8).
2. Banana leaf samples must be very dry. If necessary, leave tissue at room temperature 2-3 days to dry. Dried tissue up to 2 months old can be used for ascospore ejection.
3. Immerse leaf tissue into a 2% solution of sodium hypochlorite for 2 min. This is to reduce any surface fungi that may be present. Rinse in sterile distilled water to remove excess sodium hypochlorite.
4. Incubate leaf material with black streaks or grey spots in plastic bags containing moist tissue paper for 24–48 h.
5. Cut leaf tissue containing coalescing, necrotic lesions into approximately 5 x 5 cm.
6. Place leaf tissue into beakers that contain sterile distilled water for 20-30 min and drain excess water. Paste tissues on a 90-mm diameter Whatman filter paper and insert the filter paper in a Petri dish lid.
7. Invert a 3% water agar plate so that the upper leaf surface is closest to the agar surface. Outline the location of the leaf tissue pieces on the bottom of the agar plate with a permanent pen to assist location of germinated ascospores following incubation.
8. Leave the petri dish at room temperature for 1-2 h for the leaf piece to discharge spores onto the agar.
9. Remove the filter paper containing the leaf piece, seal the petri dish with parafilm and incubate at 25°C under 12 h alternating light and dark for 24-48 h for the spores to germinate.
10. Pick germinated spores characteristic of *Mycosphaerella* spp. off the surface of the agar using a sterile fine needle under a binocular microscope and transfer.

**Ascospore culture**

1. Look for germinated ascospores using a dissecting microscope fitted with an illuminated stage plate.
2. Select only ascospores which have one or two terminal germ tubes.
3. Using a sterile scalpel blade, dissect a small agar square containing the germinated ascospore and transfer the agar square onto a fresh PDA plate to PDA containing penicillin and streptomycin. You can also use a sterile fine needle to pick germinating spores.
4. Seal the plate with Parafilm® and incubate at 25°C for 10 days.
5. After 5-10 days, transfer colonies typical for *Mycosphaerella* spp. onto fresh PDA plates without antibiotics and incubate at 25°C for one month.
6. Ascospore cultures are transferred to V8 sporulation media for the production of conidia.
Figure 8. Left: Progressive enlargements of necrotic lesions as seen using a dissecting microscope under increasing magnification required for ascospore ejection. Right: An agar plug containing a germinated ascospore that was plated onto fresh PDA.
Other protocols that can be used to obtain pure cultures of Mycosphaerella spp. from ascospores

- Observe fresh leaf samples with lesions under a stereo microscope for the presence of pseudothecia.
- Cut leaf sections containing pseudothecia and place them in polythene bags with moist cotton wool and incubate for 24 h.
- Rinse leaf sections by immersion in sterile distilled water for 5 min.
- Staple the rinsed leaf sections onto sterile filter papers.
- Place the rinsed leaf sections previously stapled onto filter papers in the lids of Petri dishes, incubate at 25°C and allow to discharge ascospores over 3% water agar for 3 h.
- Pick individual ascospores with a sterile fine needle and transfer them to Potato Dextrose Agar (PDA) medium and incubate the plates at 25°C for 1 month.

Method 3: Conde-Ferráez et al. (2008) - Isolation from conidia
1. Use leaf tissues with streaks in the fourth stage of symptom development for conidia production.
2. Cut approximately 2 mm by 2 mm pieces and place on a stainless steel sieve.
3. Sterilise by gentle shaking, first in absolute ethanol for 30 sec, followed by 5 min in 10% (v/v) sodium hypochlorite and wash three times (2 min each) in sterile distilled water.
4. Transfer the leaf pieces to PDA plates containing 0.001 g/l each of penicillin and streptomycin and incubate for 4 - 6 days at 25°C under 12 h alternating light and dark conditions to allow production of conidia.
5. View conidia under a binocular microscope and transfer with a sterile fine needle to a fresh plate of PDA containing penicillin and streptomycin.
6. After 6 –10 days, transfer colonies typical for Mycosphaerella to fresh PDA plates using a sterile scalpel.

Generation of single spore cultures
1. Under aseptic condition, cut two pieces of culture approximately 6mm² from a healthy culture isolated on quarter strength PDA.
2. Transfer these pieces to a small 9 mL McCartney bottle containing sterile distilled water. Alternatively you can use 1.5 ml Eppendorf tubes.
3. Swirling the bottle (tubes) gently to wash the spores into solution.
4. Flame a loop to sterilise and transfer one or two loops of the spore suspension onto one side of a water agar plate.

5. Use the flamed loop to dragged through the deposit of spore suspension several times to create ‘streaks’ of spore suspension across the plate either in parallel lines or in the sixteen-streak method used in microbiology. This separates the conidia from each other to enable single, separated, germinated spores to be easily identified and excised 18-24 h later using a stereo microscope.

6. Germinated spores are observed by looking for a germ tube growing from them under stereo microscopy.

7. Once a single germinated spore has been located, use a flamed scalpel to transfer the spore to an individual plate of quarter strength PDA. Repeat this procedure 4 times for each isolated culture to ensure a pure monoconidial culture is obtained.

8. After three days, mycelial growth should be observed. Choose a culture that is healthy and showing typical growth as the representative isolate of that culture and discard the others. This culture should immediately be transferred to agar slants for short-term storage, or filter paper (see procedure below) for long-term storage.

**Pathogen identification**

*Diagnosis of Mycosphaerella species*

Sections of banana leaves showing disease are inspected for symptoms and a preliminary diagnosis made where possible based on lesion appearance. When conidia are present, morphological identifications are made. In the absence of conidia, ascospores may be used to produce conidia in culture. If a positive diagnosis cannot be made using symptomology and conidial morphology, molecular diagnosis using gel-based PCR, and species-specific primers is carried out.

**Microscopic diagnosis of disease**

The development and appearance of symptoms can differ for each of the Sigatoka diseases as a consequence of various biotic and abiotic factors such as the prevailing weather conditions, nutritional state of the plant and inoculum levels present. They will also vary due to the different levels of resistance among different banana cultivars. Therefore, it is difficult for the less experienced plant pathologist to confidently identify black or yellow Sigatoka based on leaf spot symptoms alone. A preliminary diagnosis can sometimes be made based on leaf spot symptoms, but has to be followed by
observation of fungal structures under a microscope. Although morphologically Sigatoka pathogens are very similar, there are some small but significant differences that can only be observed under a microscope on slides prepared directly from diseased leaf tissue.
Direct observation of diseased leaf tissue

Fungal structures (morphological features) can effectively be used to differentiate between *Mycosphaerella* spp (Table 5). It is therefore important that the fungus is sporulating within diseased leaf tissues. Scan a diseased leaf spot for fungal structures using a dissecting microscope. If fungal structures are easily seen a glass slide can be prepared and structures observed using a compound microscope. Alternatively, a leaf tissue with suspect lesions is cleared and fungal structures visualised *in situ*. To induce sporulation, place diseased leaf samples (stage 4 lesions) in a humid chamber (or seal diseased tissues inside a plastic bag containing sterile filter papers or paper towels soaked in sterile distilled water) overnight at 100% relative humidity at around 25°C. The high humidity will ensure a profusion of conidia and conidiophores that can be used to identify and differentiate Sigatoka pathogens.

*Note that conidia are present much earlier in *M. fijiensis* infections and can be observed as early as stage 2 lesions whereas conidia can only be seen from stage 4 lesions in *M. musicola* infections.*

Materials and Methods

- Falcon ® tubes (50 mL)
- Scissors
- Scalpel blades and handle
- Glass microscope slides and coverslips
- Dissecting and compound microscopes
- Cotton blue staining solution
- 10% KOH solution

Procedure

1. Cut 15 mm square pieces of leaf tissue (Fig. 9) containing stage 2-4 lesions approximately 1 cm in length and 2 mm in width).

2. Using a dissecting microscope, scan lesions (both top and bottom) for fungal structures. For *M. fijiensis*, the adaxial part will contain fungal structures, and for *M. musicola* or *M. eumusae*, the top side. If fungal structures are observed these can be picked off the leaf surface and placed in a droplet of cotton blue staining solution. Microscope preparations should then be observed using a compound microscope.
3. Alternatively, leaf tissues can be cleared for better observation of fungal structures. To clear the leaf tissue, place 15 mm square pieces of leaf tissue into Falcon® tubes containing 10% KOH. Leave overnight (minimum) at room temperature, then wash samples five times in sterile distilled water for 10 minutes each time. For *M. fijiensis*, place the cleared leaf tissue with the lower surface (banana leaf underside) facing up on a glass microscope slide, while for *M. musicola* or *M. eumusae* place the top side up.

4. Note that conidiophores associated with lesions can be directly observed on slides, without staining.

5. Observing conidiophores and conidia after staining: Stain cleared tissues for 1 min with a solution of 0.5% cotton blue in a mixture of lactic acid/glycerol at 3v/v; or place a few drops of cotton blue staining solution on the surface of the tissue, then cover with a glass coverslip. Seal with clear nail polish if long term storage is required. Observe conidiophores and conidia.

*Note:* To induce sporulation, leaf tissues bearing typical symptoms are sterilised and put in a humid chamber overnight, and the above steps followed to observe fungal structures.

**Figure 9.** Example of Stage 2 and 4 lesions required for identification of *Mycosphaerella fijiensis* *Mycosphaerella musicola* (anamorph *Pseudocercospora musae*) and *Mycosphaerella eumusae* (anamorph *Pseudocercospora eumusae*).
Table 5. Morphological characteristics of the anamorphs *Paracercospora fijiensis*, *Pseudocercospora musae* and *Pseudocercospora eumusae*, Sigatoka pathogens of bananas and plantains.

<table>
<thead>
<tr>
<th>Species (anamorph)</th>
<th>Conidiophores</th>
<th>Conidia</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Paracercospora fijiensis</em></td>
<td>• First appearance at early streak stage according to Fouré’s stages 2 to 3</td>
<td>• Obclavate to cylindro-obclavate, straight or curved, hyaline to very pale olivaceous, 1–10 septate, distinct basal hilum (scar)</td>
</tr>
<tr>
<td></td>
<td>• Mainly on the lower leaf surface</td>
<td>• 30–132 μm × 2.5–5 μm</td>
</tr>
<tr>
<td></td>
<td>• Emerge singly or in small groups (2 to 6), sporodochia and stromata absent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Straight or bent geniculate, pale to light brown, 0–5 septate, occasionally branched, slightly thickened spore-scars</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 16.5–62.5 μm × 4–7 μm</td>
<td></td>
</tr>
<tr>
<td><em>Pseudocercospora musae</em></td>
<td>• First appearance at spot stage (Brun’s stage 4)</td>
<td>• Cylindric to obclavato-cylindric, straight or curved, pale to very pale olivaceous, 0–8 septate, no distinct basal hilum</td>
</tr>
<tr>
<td></td>
<td>• Abundant on both leaf surfaces</td>
<td>• 10–109 μm × 2–6 μm</td>
</tr>
<tr>
<td></td>
<td>• In dense fascicles (sporodochia) on dark stroma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Straight, hyaline, mostly without septation and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• geniculation; no spore scars</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 5–25 μm × 2–5 μm</td>
<td></td>
</tr>
<tr>
<td><em>Pseudocercospora eumusae</em></td>
<td>• First appearance at spot stage</td>
<td>• Subhyaline to pale olivaceous, thick-walled, smooth, subcylindrical, apex</td>
</tr>
<tr>
<td></td>
<td>• Mainly on the upper leaf surface</td>
<td>• obtuse, base subtruncate, straight to variously curved, 3–8 septate</td>
</tr>
<tr>
<td></td>
<td>• In dense fascicles on brown stroma</td>
<td>• 30–50 μm × 2.5–3 μm</td>
</tr>
<tr>
<td></td>
<td>• Subcylindrical, hyaline or pale brown below, 0–3 septate, straight to geniculate-sinuous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 10–25 μm × 3–5 μm</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Wardlaw (1972), and Crous and Mourichon (2002).
Molecular Diagnosis of *Mycosphaerella* species

Molecular diagnosis is used to either confirm morphological results or cases where a positive diagnosis cannot be made using symptomology and conidial morphology. Polymerase chain reaction with species specific primers can be used to unequivocally detect, identify and differentiate *Mycosphaerella* species. PCR can be conducted on DNA extracted directly from diseased leaf tissues or from pure cultures of the fungus (Fig. 10). PCR techniques for detecting and identifying *Mycosphaerella* species, are outlined below.

**Detection and identification of fungi associated with Sigatoka using PCR**

![Work scheme for PCR identification of Fungi associated with Sigatoka disease of banana using species-specific primers.](Image)

**Figure 10.** Work scheme for PCR identification of Fungi associated with Sigatoka disease of banana using species-specific primers.
Detection and identification of *Mycosphaerella* directly from diseased leaves

**DNA extraction**

If the objective is to detect and identify *Mycosphaerella* species associated with banana disease, this method should be used as it is fast and avoids the length stages of pathogen isolation and establishment of pure cultures. For direct detection and identification of fungi associated with Sigatoka, DNA can be extracted from leaf samples, using a variety of protocols and PCR conducted with species specific primers. To enrich for fungal DNA, put symptomatic leaf tissues in a humid chamber (avoid using necrotic lesions) over night and the leaf tissues used for DNA extraction. Most of the protocols outlined below can be used to extract DNA from leaf samples. The method of Berendzen *et al.* (2005) is outlined below:

*DNA extraction directly from diseased leaf samples (Berendzen *et al.*, 2005)*

This method uses a sucrose solution for direct extraction of DNA from diseased plant materials. To enrich the samples for fungal DNA, it is advisable to put the samples in a humid chamber (created by putting sterile cotton wool or filter papers soaked in sterile distilled water over night). This will encourage the fungus to grow and the leaf tissues can be used for direct DNA extraction.

**Procedure**

1. Place approximately 10 mg of leaf tissue directly in 200 μl sucrose solution.
2. Grind at room temperature or on ice using a pipette tip or pestle.
3. Heat sample to 99–100°C for 10 min
4. Briefly spin at 2000–6000 g for 5 sec.
5. Place sample on ice until PCR.
6. 1 μl of the supernatant is used for PCR, avoiding debris.

**The Sucrose Prep**

**Sucrose Solution:**

- 50 mM Tris-HCl pH 7.5
- 300 mM NaCl
- 300 mM sucrose
**DNA extraction from pure fungal cultures**

This method should be used if genetic characterisation of the different *Mycosphaerella* spp. is desired. Pure cultures of each isolate must be established and preserved, and then used for identification and genetic characterisation purposes. High quality DNA is essential to carry out molecular procedures. The selection of DNA extraction protocol depends on the equipment and reactive available in the laboratory. Mycelia or spores from single-spore isolates cultures are obtained from solid (media with agar) or liquid (culture in broth) media. DNA extraction can be carried out by different protocols or by using specific DNA extraction kits following indications of the kit employed.

**Growth and preparation of fungal mycelia for DNA extraction**

Cultures stored at 4 °C on PDA slants are subcultured on PDA and incubate at 25 °C under 12 h alternating light and dark conditions. An actively growing culture is required to produce fungal mycelium or spores for DNA extraction.

**Production of fungal mycelium**

**Method 1: Production of fungal mycelium in Potato dextrose broth (PDB)**

1. Grow *M. fijiensis* cultures for DNA extraction in potato dextrose broth (PDB) liquid medium.
2. Sterilise conical flasks (250 ml) containing approximately 50 ml of PDB by autoclaving and inoculate the medium with mycelial fragments of the isolates from cultures on PDA.
3. Incubate the cultures on an orbital shaker at 100 rpm at room temperature for 18 - 28 days depending on the growth rate of the isolates.
4. Filter the grown cultures under vacuum through a buchner funnel and collect mycelia on Whatman No.2 filter paper. Freeze the mycelia at -20°C for 3 - 5 h and then freeze-dry.
5. Grind freeze-dried mycelia in liquid nitrogen in sterile mortar and pestle. Dispense approximately 150 mg of ground mycelia into 1.5-ml microcentrifuge tubes and store at -20 °C until ready for DNA extraction.
Production of fungal mycelium V8 juice medium (Mahuku et al., 2004)
1. Prepare 60 mL of liquid V8 juice in Erlenmeyer flasks (200 mL), autoclave to sterilise.
2. Inoculate the flasks with ten 1-cm discs removed from actively growing monosporic cultures.
3. Incubate the cultures at 25°C on a rotary shaker (115 rpm) for 7-10 days.
4. Harvest mycelia by filtration through cheesecloth, blotted dry with sterile paper towels, and use immediately for DNA extraction.
5. Mycelium can be freeze-dried as outlined above and used for DNA extraction.

DNA isolation from mycelia
Method 1: DNA extraction from mycelium (Surridge et al., 2003)
1. Place 1 g of fresh tissue or fungal mycelium in an eppendorf tube and grind with 10µg sterile river sand in 500 µL of DNA extraction Buffer (DEB: 200 mM Tris-HCL (pH 8), 150 mM NaCl, 25 mM EDTA (pH 8), 0.5% SDS).
2. Add 200 µL DEB to the suspension with 500 µL of phenol and 300 µL chloroform.
3. Vortex and centrifuge for 60 min at 10000 rpm.
4. Transfer the supernatant to a new tube and add 500 µL of phenol and 500 µL of chloroform.
5. Centrifuge for 5-10 min at 10 000 rpm. Repeat the phenol/chloroform stage until the interface is clean.
6. Add 500 µL more of chloroform and centrifuge for 5 min at 11 000 rpm.
7. Transfer supernatant to a new tube and add double the volume of 100% ethanol and mix.
8. Allow DNA to precipitate overnight at 4°C and pellet by centrifugation.
9. Pellet by centrifugation for 30 min at 11 000 rpm.
10. Wash pellets with 500 µL 70% ethanol, dry and resuspend in 100 µL sterile distilled water and 3 µL RNase (2.5 µM).
Method 2: TES DNA Extraction Protocol (Mahuku, 2004)

1. Transfer fresh mycelium (150 mg) to a sterilised 1.5-mL Eppendorf (micro centrifuge) tube containing 300 µL of TES extraction buffer (0.2 M Tris-HCl [pH 8], 10 mM EDTA [pH 8], 0.5 M NaCl, 1% SDS) and acid-washed, sterilized sea sand or 0.5-mm glass beads.

2. Macerate mycelium for 2 min with a hand-held disposable homogenizer that fits the 1.5-mL micro centrifuge tube.

3. Vortex samples for 30 s and add an additional 200 µL of TES extraction buffer containing proteinase K (final concentration of 50 µg/mL).

4. Vortex to thoroughly mix and place tubes in a water bath at 65°C for 30 min.

5. Add one-half volume (250 µL) of 7.5 M ammonium acetate.

6. Mix and incubate the samples on ice or at ~5°C in the refrigerator for 10 min.

7. Centrifuge for 15 min at 20,800g (13000rpm).

8. Transfer the supernatant to a new tube and add an equal volume (500 µL) of ice-cold isopropanol.

9. Incubate tubes at −20°C for 1-2 h.

10. Centrifuge for 10 min at 20,800g (13000rpm) to pellet the DNA.

11. Decant the supernatant and wash DNA pellet with 800 µL of cold 70% ethanol.

12. Turn tubes upside-down on clean sterile paper towels for 10-15 min to air-dry DNA.

13. Elute DNA from the pellet with twice-repeated extractions with 250 µL of 1×TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA), each time centrifuging to avoid collecting pelleted polysaccharides.

14. Transfer DNA solution to a 1.5-mL micro centrifuge tube, add 5 µL of RNase A (20 mg/mL), and incubate at 37°C for 60 min.

15. Recover DNA and air-dry as described above.

16. The dry DNA pellet can be shipped to other laboratories for analysis or dissolved in 100 µL of 1× TE buffer.

17. Suspend pellet in 100µL of Nuclease-free water, vortex. Run a DNA test gel using 1% agarose. Determine DNA integrity with a Nanodrop spectrophotometer (for high quality dsDNA, A$_{260}$/A$_{280}$ range is 1.7 to 1.9), OR run 1% test gel.

18. Store DNA samples at -20°C.

19. Treat samples by adding 2µL of RNase A (20µg/mL) and incubate at 37°C for 1hr.

Notes

- Mycelium can be lyophilised or frozen in liquid nitrogen before grinding with a hand-held pestle that fits in a 1.5-mL micro centrifuge tube.
- If the DNA pellet is loose, centrifuge the sample for 3 min at 3800 g.
Method 3: DNA extraction Protocol (Modified CTAB) - Lodhi et al. (1994),
The CTAB (Cetyl-trimethyl ammonium bromide) method of Lodhi et al. (1994), omits - mercaptoethanol from the extraction buffer, and chloroform: isoamylalcohol (24: 1 v/v) cleaning step included.

Procedure
1. Put approximately 150 mg of ground mycelia into a 1.5-ml micro centrifuge tube and add 750 µl of extraction buffer into the tube.
2. Mix the contents of the tubes well by repeated inversion and place the tubes in a heated block at 65 °C for 30 min.
3. Add 600 µl of chloroform:isoamylalcohol (24:1v/v) and mix the samples gently until cloudy and centrifuge at 8000 rpm for 30 min.
4. Transfer the supernatant solutions to new 1.5ml tubes and add 25 µl of 25 mg/ml ribonucleaseA (RNase).
5. Mix the tubes contents as above and place in a heated block at 55 °C for 30 min.
6. Add a further 600 µl of chloroform:isoamylalcohol (24:1 v/v) to each tube, mix and centrifuge as above.
7. Transfer the supernatant solutions to new tubes, add half the volume of 5 M NaCl followed by 600 µl of 95 % (v/v) cold absolute ethanol (-20 °C) to precipitate the DNA.
8. Place the tubes in the freezer at -20 °C for 15 - 20 min; pellet the DNA by centrifugation at 6000 rpm for 10 min.
9. Wash the DNA pellets with 500 µl of 75 % (v/v) ethanol, vacuum-dry and suspend the dried pellet in 200 ml of TE (10 mM Tris, 1 mM EDTA) buffer.
10. Store the DNA samples in the freezer at -20 °C.

Extraction buffer pH 8.0(To make 1 Litre)
- 12.1 g Tris,
- 7.44g EDTA,
- 87.66 g NaCl,
- 10 g CTAB (adjust to pH 8.0 with conc. HCl)
- 50 µl of 20 % (w/v) polyvinyl pyrrolidone (PVP).
**PCR Amplification**

The Sigatoka PCR assay have been designed to amplify sections of different genes, e.g. ribosomal DNA (Johanson and Jeger, 1993); actin and β-tubulin genes (Arzanlou et al., 2007). Using these regions allows good species differentiation. Use of ribosomal DNA also gives good sensitivity as is present in high copy number in the pathogen genome.

**PCR protocol**

**Primers**

To differentiate between species of *Mycosphaerella*, DNA extracted from pure fungal cultures or infected leaf material is amplified using species-specific primers (Table 6, 7).

**Table 6. Mycosphaerella** species-specific primers (Arzanlou et al., 2007)

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Sequence (5’ → 3’ )</th>
<th>Target</th>
<th>Location</th>
<th>Primer combination / Fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTFa</td>
<td>TCCAACCGTGAGAAGATGAC</td>
<td>General Actin</td>
<td>ACTF/ACTR (820 pb)</td>
<td></td>
</tr>
<tr>
<td>ACTRa</td>
<td>GCAATGATCTTGACCTTCAT</td>
<td>General Actin</td>
<td>ACTF/ACTR (820 pb)</td>
<td></td>
</tr>
<tr>
<td>MFactF</td>
<td>CTCATGAAGATCTTGCTGAG</td>
<td>M. fijiensis Actin</td>
<td>ACTR/MFactF (500-bp)</td>
<td></td>
</tr>
<tr>
<td>MMactF2</td>
<td>ACGGCCAGGTCATCACT</td>
<td>M. musicola Actin</td>
<td>MMactF2/MMactRb (200-bp)</td>
<td></td>
</tr>
<tr>
<td>MMactRb</td>
<td>GCGCATGGAAACATGA</td>
<td>M. musicola Actin</td>
<td>ACTF/MMactRb (630-bp)</td>
<td></td>
</tr>
<tr>
<td>MEactR</td>
<td>GAGTGCGCATGCGAG</td>
<td>M. eumusae Actin</td>
<td>ACTF/MMactR (820-bp)</td>
<td></td>
</tr>
</tbody>
</table>
PCR conditions

PCR reactions are carried out in 25 µl volumes

Each reaction mix contains:
1× PCR buffer,
1.5 mM MgCl₂,
60 µM dNTPs,
0.2 µM primers,
1.5 U of Taq DNA polymerase,
1 ng of genomic DNA.

PCR cycle conditions are as follows: 1 cycle of 95°C for 5 min, followed by 36 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s; with a final elongation cycle at 72°C for 7 min.

Table 7. *Mycosphaerella*-species specific primers (Johanson and Jeger, 1993)

<table>
<thead>
<tr>
<th>Primers and Probes</th>
<th>Nucleotide Sequence 5’ to 3’</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF137</td>
<td>GGCGCCCCCGGAGGCCGTCTA</td>
<td>rDNA</td>
</tr>
<tr>
<td>MM137</td>
<td>GCGGCCCCCGGAAGGTCTCCTT</td>
<td>rDNA</td>
</tr>
<tr>
<td>R635</td>
<td>GTCCGTGTTTCAAGACGG</td>
<td>rDNA</td>
</tr>
</tbody>
</table>

PCR reactions are carried out in 50-µl volumes in 0.5-ml microcentrifuge tubes.

- Each reaction mix contains
- 0.02 U Super Taq polymerase,
- 1 Super Taq buffer (100 mM Tris-HCl, pH 9.0)
- 15mM MgCl₂,
- 500 mM KCl,
- 1 % Triton-X-100,
- % (w/v) stabiliser),
- 0.4 mM dNTPs
- 0.6 µM each of MF137 or MM137 and R635
- ng DNA template.
Carry amplification out in a thermal cycler programmed to give 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 65 °C for 1 min and 72 °C for 2 min, with a finally single cycle of 72 °C for 5 min.
Agarose gel electrophoresis

- Mix 10-l volume of each PCR product with 2 l bromophenol loading dye.
- Separate the DNA fragments by electrophoresis at 3.3 V/cm in a 1.5 % (w/v) agarose gel in 0.5× TBE buffer.
- After electrophoresis, stain the gel in 1 g/ml ethidium bromide for 30 min in order to visualize the PCR products under 302nm UV light.
- Estimate the size of the products produced on the gel using a DNA size marker (Kb DNA ladder).

Field and greenhouse trials

Field screening of banana plants follows natural field infections. For greenhouse experiments and laboratory screening using a detached leaf assay, inoculum needs to be prepared for pathogenicity testing.

Greenhouse screening of banana and plantain genotypes

Starting material

- Banana plants derived from in vitro multiplication (tissue culture or suckers).
- Inoculation done 10 weeks after the beginning of acclimatisation (plant size 40–60 cm, 10 leaves).
- For the inoculation technique on leaf pieces, banana plants have to be grown under optimum physiological conditions before sampling.

Inoculum preparation

- From a well sporulating fungus in PDA, cut a agar plug and put in a test tube with 5 mL distilled water and 3 mL Tween® 0.05%.
- Vortex test tube for 1 min to release mycelium fragments and spores from the agar plug.
- Using a Pasteur pipette put 15 drops of the solution on a Petri dish with V8-juice medium.
- Spread the solution over the medium with a sterile spatula.
- Put petri dishes in an incubator for 7 days at 26° C under artificial light conditions.
- After 7-10 days, add a solution of 10 mL of water with Tween® 0.05% on, scrap the surface with a spatula to release the spores.
- Filter through two layers of cheese cloth.
- Estimate spore concentration of using a haemacytometer.
- Adjust spore concentration using sterile distilled water with 3 mL Tween® 0.05%.
Artificial inoculation on banana plants

- Inoculate the youngest leaf using an artist air brush (normally used for painting), using a spore suspension (1 x 10^5 spores /ml)
- Spray approximately 1 mL of suspension on the lower surface of the youngest leaves using a microsprayer at constant pressure (1.5 kg·cm^{-2})
- For *M. fijiensis*, inoculate the adaxial part of the leaf, whereas for the other *Mycosphaerella* species, inoculate the top part of the leaf
- Allow the inoculated area to dry for 1 to 2 hrs, then put plants into a humid chamber with humidity >80%
- Place inoculated plants in a climatic chamber with relative humidity allowing a saturated atmosphere, at 25°C, and under a 12 hr/12 hr photoperiod
- After 1 week, reduce the relative humidity to 80%
- Monitor plants for infection by studying the inoculated leaf for symptoms at a 2-day interval

*Note:* first symptoms appear after 10–15 d of incubation, and the time from the first to final necrosis lesions differs according to the level of host susceptibility: from 20–25 d for a susceptible plant to 60–70 d for a highly partially resistant cultivar (Figure 6).

Laboratory screening using the detached leaf assay

- Select the youngest fully mature leaf from plants (if necessary the second youngest leaf may be used as well), cut it and bring it back to the laboratory. To conserve the leaf until needed, put the stem in water.
- Cut opened leaves into large pieces that can easily be cleaned in a 1-L beaker.
- Surface-sterilise leaf pieces in 1% NaOCl solution for 90 s, and wash in 5 to 6 changes of sterile distilled water.
- Aseptically cut leaf pieces into segments of 6×6 cm, and place them in a Petri dish with the upper leaf surface face down on the survival medium (0.4% water:agar and 50 mg benzimidazole (Sigma product, ref. B–9131)-L–1 in Petri dishes (100 mm in diameter).
- Can also use 1% technical agar amended with 5mg/l of gibberellic acir (1% technical agar; autoclave, and add 5 mg/l of gibberellic acid immediately before dispensing in Petri dishes. (Gibberellic acid at this concentration is able to maintain a green colour in banana leaves for 45 to 52 days (Twizeyimana et al., 2007).
- Place a piece in plastic Petri dishes with the adaxial side on 1% agar technical medium amended with gibberellic acid.
- Add a drop of 1% Triton X-100 added to the spore suspension prior to inoculation.
• To inoculate, add two droplets of inoculum per leaf piece by pipetting 40 μl droplets of spore suspension (5 × 10⁵ conidial/ml) onto the abaxial side of the leaf.
• Seal Petri dishes with parafilm and incubate at 25°C with a 12:12 hour light:dark cycle.

**Caution:** it is very important to avoid any condensation in the petri dish to keep leaf pieces alive (Figure 7). Use a climatic chamber with a circular movement of fresh air. Note: first symptoms appear after 18–20 d of incubation, and the time from the first to final necrosis lesions differs according to the level of host susceptibility from 30 d for susceptible plants to 60–70 d for a partially resistant cultivar.

**Scoring of Sigatoka trials**

Start observing for symptoms 2 days after inoculation and can last for 32 days. Long days of assessment are required for genotypes that have higher levels of resistance to Black Sigatoka disease. Record data on incubation time, disease severity, symptom evolution time and symptom stages as described above were recorded.

**Disease assessment parameters**

- **Incubation time** is defined as the time between inoculation and appearance of the first symptoms (Stage 1),
- **Disease severity** (% of leaf area infected),
- **Symptom evolution time** is defined as the number of days between Stage 1 and occurrence of mature lesions (Stage 6).
### Field Scoring of Sigatoka Leaf spots

**Proposed field evaluation form**

<table>
<thead>
<tr>
<th>Date:</th>
<th>GPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>District, Subcounty:</td>
<td>GPS</td>
</tr>
<tr>
<td>Parish, Village:</td>
<td>Altitude</td>
</tr>
</tbody>
</table>

**Management practice:**

<table>
<thead>
<tr>
<th>Plant no.</th>
<th>Cultivar</th>
<th>Sigatoka type (Black/Yellow)</th>
<th>No. of standing leaves (NSL)</th>
<th>Youngest leaf spotted (YLS)</th>
<th>Disease severity</th>
<th>Index of non-spotted leaves (INSL)</th>
<th>Management practice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NB:**

- Youngest leaf spotted (YLS) will be assessed according to the method of Stover and Dickson (1970), which involves the monitoring of the youngest leaf (from the top of the plant) bearing at least 10 black Sigatoka necrotic lesions.
- Disease severity index (SI) method involves a visual estimation of the necrotic area per plant, scored according to a six-disease grades scale. The SI for one plant corresponds to the sum of the scores per leaf.
Table 7. Summary of the different lesion stages associated with yellow and black Sigatoka leaf spot diseases of banana.

<table>
<thead>
<tr>
<th>Lesion Stage</th>
<th>Black Sigatoka</th>
<th>Yellow Sigatoka</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>Small pigmented spot of white or yellow, similar to yellow Sigatoka Stage 1.</td>
<td>Very small light green dot or dashes of approximately 1 mm in length.</td>
</tr>
<tr>
<td>Stage 2</td>
<td>Brown streak, visible on underside of leaf, later visible on leaf upper surface as yellow streak; colour changes progressively to brown, then black on upper leaf surface</td>
<td>Light green streak several millimetres long.</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Enlarged stage 2, streaks become longer</td>
<td>An elongated rusty brown spot with a poorly defined border.</td>
</tr>
<tr>
<td>Stage 4</td>
<td>Appears on leaf underside as brown spot, as a black spot on upper leaf surface</td>
<td>A mature, more elliptical spot with a dark brown sunken centre; often surrounded by a yellow halo, conidiophores and conidia are produced at this stage.</td>
</tr>
<tr>
<td>Stage 5</td>
<td>Elliptical spot is totally black on the underside of the leaf, surrounded by a yellow halo</td>
<td>Spot has developed a grey, dried out centre and peripheral black ring which is evident even after the leaf has dried out.</td>
</tr>
<tr>
<td>Stage 6</td>
<td>Centre of spot dries out, turns grey and is surrounded by a well-defined margin and a bright yellow halo</td>
<td></td>
</tr>
</tbody>
</table>
Table 8. Disease assessment key (0-6 scale) for estimating the infection caused by *Mycosphaerella* species on a single plant (Disease Severity Index).

<table>
<thead>
<tr>
<th>Rating scale</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No visible symptoms of the disease</td>
</tr>
<tr>
<td>1</td>
<td>Less than 1% (only streaks or up to ten spot of the leaf with disease symptoms)</td>
</tr>
<tr>
<td>2</td>
<td>1 to 5% of the leaf area with symptoms</td>
</tr>
<tr>
<td>3</td>
<td>6 to 15% of the leaf area with symptoms</td>
</tr>
<tr>
<td>4</td>
<td>16 to 33% of the leaf area with symptoms</td>
</tr>
<tr>
<td>5</td>
<td>34 to 50% of the leaf area with symptoms</td>
</tr>
<tr>
<td>6</td>
<td>51 to 100% of the leaf area with symptoms</td>
</tr>
</tbody>
</table>

Data to record in greenhouse experiment

1. **Incubation time**: Time between infection with pathogen and appearance of symptoms
2. **Leaf area infected (%)**: 
3. **Symptom evolution time**: Time from appearance of first symptoms to development of mature spots
4. **Stage of symptoms**: Scale 1-6 as described above
5. **Latent period**: This is the time taken by fungus to start producing mature pseudothecia and ascospores.
Useful reading


Stover, R.H. 1980. Sigatoka leaf spot diseases of bananas and plantains. Plant Disease 64:


The banana weevil

Background

The banana weevil, *Cosmopolites sordidus* (Germar) is an important pest on bananas and plantains (*Musa* sp.). The adult weevil is black and measures 10-15 mm. It is free living, though most commonly found between leaf sheaths, in the soil at the base of the mat or associated with crop residues. The weevil is nocturnally active and very susceptible to desiccation. Adults may remain at the same mat for extended periods of time, while only a small proportion will move >25 m within 6 months. The weevils rarely fly. Dissemination is primarily through infested planting material. Many adults live for 1 year, while some survive up to 4 years. On moist substrates, the weevil can survive without feeding for several months. The sex ratio is 1:1. Oviposition rates of more than 1 egg/day have been recorded, but most commonly, oviposition has been estimated at 1 egg/week. The female places its white, oval eggs singly into holes made by the rostrum. Most oviposition is in the leaf sheaths and rhizome surface. Flowered plants and crop residues are favoured stages for oviposition.

The emerging larvae preferentially feed in the rhizome, but will also attack the true stem and, occasionally, the pseudostem. The larvae pass through 5-8 instars. Pupation is in naked cells near the surface of the host plant. Developmental rates are temperature dependent. Under tropical conditions, the egg to adult period is 5-7 weeks. Egg development does not occur below 12°C; this threshold may explain why the weevil is rarely encountered above 1600 masl. Adult banana weevils are attracted by volatiles emanating from host plants. Cut rhizomes are especially attractive.

Symptoms

Banana weevil attack has been reported to interfere with root initiation, kill existing roots, limit nutrient uptake, reduce plant vigour, delay flowering and increase susceptibility to other pests and diseases (Fig. 11). Loss of more than 40% of the plant crop to banana weevil has been recorded. Yield reductions are caused by both plant loss (plant death, rhizome snapping, toppling) and lower bunch weights. Thus the banana weevils have been contraindicated in the short plantation life of bananas. The population of the weevil builds up slowly and damage becomes increasingly important in successive crop cycles/ratoons (Figure 12).
Figure 11. Symptoms of banana weevil.

Figure 12. Cumulative weevil damage and yield loss in successive banana cycles.

Sample collection

Setting weevil traps

- Identify a weevil infested banana plantation (usually the field is older than 5 years).
- Cut the banana pseudostem into pieces of about 30 cm long (usually the lower part of the stem of freshly harvested plants make the best traps) (Fig. 13).
- Split the pseudostem pieces longitudinally into two discs.
- Place the split pseudostems discs around the mats (preferably on a stump).
- Leave the traps (split pseudostems discs) for 3 days.
- Collect the trapped weevils in a 30 litre bucket.
Accurate notes must be taken for each sample, including:

- Sample number (one sample number per plant).
- Date of collection.
- Age of plantation.
- Notes about the plantation (Location, village, GPS coordinates, name of dominant banana genotype, main intercrop and the source of the planting material).
- Collectors’ names, and the required phytosanitary certificate/importation permit.
- Other useful observations like plantation management practices, chemical control (use of insecticides, note the type), cultural control and biological control measures.

Maintenance of healthy cultures of the banana weevils:

- Banana weevil is cultured on a pared rhizome of a highly susceptible variety like Atwalira.
- After laying eggs on rhizomes for 5-7 days, rhizomes are transferred to another 30l bucket.
- Rhizomes are sprayed with water to maintain an appropriate relative humidity of 70-80% using a plastic watering can until adult weevils emerge (after about 50 days).
- To ensure raise weevils of different ages, fresh cultures can be established at monthly intervals.

**Figure 13.** Split pseudostem trap near the mat (A), Banana weevils trapped after 3 days (B).
**Banana weevil identification**

The adult banana weevil is dark brown to black in colour (Fig 14), has a long snout and a hard shelled-body. It can live for some time without food but will die within 2 days in a dry environment. It thrives well in moist places. The weevil densities are higher in mulched areas. Its life span is up to 2 years.

**Sexing banana weevils**

- To ensure a continuous cycle, banana weevils are infested in a 1:1 (male: female) ratio.
- Use a microscope or hand lens to view punctation on the rostrum to distinguish between male (fully punctated rostrum) and female weevils (less than half rostrum punctated).
- Confirm the sex by checking for curving of the last segment of abdomen the male weevils and flat abdomen for the female weevil.

![Figure 14. The adult banana weevils.](image)

**Field and greenhouse trials**

Banana weevil resistance has been attributed to biophysical factors like rhizome diameter, resin/sap production, rhizome dry matter content, rhizome hardness, and suckering ability (number of suckers). Generally antibiosis (factors affecting larval performance) rather than antixenosis (attraction) appear to be the most important resistance mechanism in banana. A wide range of damage assessment methods exist and it is critical to know what types of damage best reflects response of genotypes to banana weevil and how it relates with yield loss.
Reference cultivars for screening for weevil resistance

Clones that could be included during the evaluation of new and improved hybrids for their response to banana weevils are:

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Resistant</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yangambi KM 5 (ITC1123)</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Culcatta 4 (ITC 0249)</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>EA-AAA (Kibuzi)</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>EA-AAA (Mbwazirume)</td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

Artificial infestation of banana weevils in field trials (cultivar screening or yield loss)

- Keep the weevils in dark room with red light on for at least 15 hr prior to being used.
- Release/infest 10 banana weevils per plant, 9 months after planting, in the ratio of 1:1 female to male to make a population density of about 25 000 weevils/ha.
- Determine establishment and multiplication of the weevils, 6 months after releasing banana weevils by laying traps on mats in at least 2 blocks and count the number of weevil per trap.

Scoring of banana weevil trials

Rhizome damage assessment

- Damage assessment requires destructive sampling and is done on rhizomes 0–15 days after harvesting.
- Cut a transverse cross section at the collar (upper cross section).
- Score weevil damage (galleries) as percentage damage on the upper cross-section (at collar area) for both the inner rhizome (central cylinder) and the outer rhizome (cortex).
- Cut another transverse cross section at 10 cm below the collar (lower cross-section).
- Score weevil damage (galleries) as percentage damage on the lower cross-section for damage on both the inner rhizome (central cylinder) and the outer rhizome (cortex).
- Calculate the total damage as the average of cross section damage of the central cylinder and cortex.

Mat disappearance

- Count the total number of mats of each genotype in the trial at an interval of 6 month beginning 6 months after planting.
- Compare the counts over time to verify the effect of banana weevils on stability of the genotypes.
Bioassay for evaluating larval performance on rhizome discs for various cultivars

- The larval stage that is the most actively feeding and destructive stage of the banana weevil.
- Weevils are cultured on rhizomes of a susceptible cultivar (Atwalira) in a 30-L bucket.
- Feed the weevils on fresh peeled pseudostem of the susceptible cultivar where eggs are laid after 2-3 days.
- Collect the eggs from the pseudostem and sterilise them with 25 ml of distilled water supplemented with 5 ml ethanol (20% ethanol) and 2-3 drops of JIK on a Petri dish.
- Spread the eggs on a moistened kitchen towel tissue in a Petri dish using painters brush.
- Store the eggs at room temperature on the Petri dishes and wetted daily to avoid desiccation for about 3-4 days.
- Prepare rhizome sections (4x2x1 cm) from banana genotypes to be screened and place them in Petri dishes (the rhizome sections are obtained from flowered plants).
- Bore the rhizome section with four holes on one side.
- Place 6-7 days old banana weevil egg singly in each hole, then cover with rhizome tissue and seal with cling film. Label the Petri dish.
- At 6-7 days, the red heads of the developing weevil larva are visible in the eggs and close to hatching.
- Using eggs at this stage are used to minimise the potential injuries to larva that could result from the soft ‘camel hair’ brushes that are used for their transfer.
- This also ensures that only viable eggs are selected for the study.
- Place the Petri dishes in plastic boxes and keep at room temperature for eight days after which the larvae are removed for measurements.
- After 8 days, the larvae are retrieved from the rhizome section and head capsule width, body length, body weight and larval mortality are recorded.
- This procedure is repeated three times and these are taken as replications over time.
Useful reading


Banana nematodes

Background

Nematodes are a diverse group of worm-like animals. They are found in virtually every environment, both as parasites and as free-living organisms. They are generally minute and require a microscope to observe them. This guide focuses specifically on plant parasitic nematodes, which are very small or microscopic, can cause significant damage to crops, and are extremely widespread.

Because nematodes are difficult or impossible to see in the field, and their symptoms are often non-specific, the damage they inflict is often attributed to other, more visible causes (Fig. 15). Farmers and researchers alike often underestimate their effects. A general assessment is that plant parasitic nematodes reduce agricultural production by approximately 11% globally, reducing production by millions of tonnes every year. The amount of damage nematodes cause depends on a wide range of factors, such as their population density, the virulence of the species or strain, and the resistance (ability of the plant to reduce the population of the nematode) or tolerance (ability of the plant to yield despite nematode attack) of the host plant. Other factors also contribute to a lesser extent, including climate, water availability, soil conditions, soil fertility, and the presence of other pests and diseases.

Although quite a number of species may occur on banana, just a few key species are of importance. *Radopholus similis* or the burrowing nematode is generally seen as the most damaging species on banana across banana growing regions, but in East and Central Africa the nematodes of economic importance include *R. similis*, *Pratylenchus coffeae* and *P. goodeyi* or root lesion nematodes, *Helicotylenchus multicinctus* or spiral nematodes and *Meloidogyne* spp. or root knot nematodes. *Rotylenchulus reniformis* or the reniform nematode may occur locally in patches. A combination of species is most likely to be encountered on banana however.

Symptoms

*Pratylenchus* spp., *R. similis* and *H. multicinctus* infection of roots all result in necrotic lesions parallel to the root axis, and eventual decomposition and death of the root (Fig. 16). *Pratylenchus* spp. and *R. similis* damage will often extend to the central stele.
while *H. multicinctus* tends to remain on the outer edges of the root cortex. As the nematodes often occur together though, so the damage symptoms are combined. Migration of the nematodes through the cortical cells results in the extensive necrosis and visible lesions when roots are sliced. Heavily infected root systems are stunted with numerous dead roots; root surfaces may be cracked or have irregular root swelling. Roots infected with *Meloidogyne* spp. will often have disfigured swollen roots, which also become necrotic and decompose into blackened dead roots. Above-ground symptoms results from root disfunction and reduced water and nutrient uptake with stunted, chlorotic plants typical. Affected plants will also have a reduced canopy leaf cover, thinner stems, take longer to fruit and yield smaller bunches. Heavily infected root systems become unable to provide plant anchorage and characteristic toppling over of banana plants is typical of nematode damage (Fig. 17).

**Figure 15.** Stunting/reduced height of plantain (plants on left) caused by *Pratylenchus coffeae*. 
Figure 16. Banana roots showing extended root lesioning (necrosis) caused by *Radopholus similis*.

Figure 17. Toppling over of banana caused by *Radopholus similis*.
Disease cycle and epidemiology

*Radopholus similis* males may comprise 0 - 50% of the population although male nematodes do not feed. The burrowing nematode infects at or near the root tip and resides almost exclusively in the root cortex, although they are also known to damage the banana stele. The nematode remains within the root until overcrowding and root decay causes them to migrate. Population development is host dependant. In banana, females begin to lay eggs at an interval rate of nearly 5/day at optimum temperature; juveniles may hatch in 2-3 days. Most populations of *R. similis* reproduce best at intermediate (25°C) or high (30°C) rather than lower (15–20°C) temperatures. *R. similis* is highly polyphagous, attacking over 250 plant species.

*Pratylenchus goodeyi* and *P. coffeae* deposit eggs at rates up to two/day, mainly in root tissue but also in soil along the root surface. All life stages of lesion nematodes can be isolated from both soil and roots. Juveniles sometimes feed ectoparasitically on plant root hairs, but more commonly are found mainly in the root cortex. Cell death occurs when nematodes migrate through the cell or pause to feed. Life cycles may be completed in 3-4 weeks, although at higher altitudes and lower temperatures, life cycles become longer. Both species characteristically have quite a wide host range, especially *P. coffeae*.

*Helicotylenchus multicinctus* occurs frequently in roots that are infected with other nematode species. As with the burrowing and lesion nematodes, the spiral nematode enters the root and migrates through the root as it feeds and causing cell damage and death, usually on the outer edges of the root. When extracted, *H. multicinctus* are distinguished from other nematodes by their longer stylets and by the letter C shaped body when killed. *Radopholus similis, Pratylenchus* spp. and *Meloidogyne* spp. are straight when at rest.

On banana, *Meloidogyne* spp. cause galls and swellings on primary and secondary roots. Sometimes, the root tips are invaded and there is little obvious galling or swelling, but root growth is stopped and new roots proliferate just above the infected area. Infected plants may have a much lower number of secondary and tertiary roots and root hairs. Root knot nematodes are sedentary, and after entering the root, usually just behind the root tip, they establish a feeding site and remain in situ for the duration of their life cycle, which may be as little as 20 days.
Sample collection

Soil samples to represent different banana cropping sequences will be collected. In each cropping sequence, a spade will be used to collect soil and roots from five randomly distributed plants by digging 30x30 cm holes ~1 m distance from the plants. The five sub-samples taken from each field will then be mixed homogeneously to constitute a composite sample. Any soil clods in each composite sample will be carefully broken up with the fingers and sieved gently through a coarse sieve to remove stones or debris. Approximately 500 g of soil will be taken, placed in a clearly labelled plastic bag, sealed and then stored in a cool box or under cool conditions, but out of direct sunlight or heat. The samples will be transported to the laboratory in a cool box and stored at 10°C for nematode assay. Soil samples will be processed as soon after collection as possible, within a few days after the collection.

Nematode isolation

Nematode extraction and assay

Extraction tray method (Baermann funnel technique)

This method (or variations of it) is sometimes also called the modified Baermann technique, the pie-pan method, or the Whitehead tray method (Fig. 18).
Figure 18. Extraction tray method (Baermann funnel technique).

Advantages:
- Specialist equipment is not required
- It is easy to adapt to basic circumstances using locally available materials
- It extracts a wide variety of mobile nematodes
- It is a simple technique.

Disadvantages:
- Large and slow moving nematodes are not extracted very well
- The extractions can sometimes be quite dirty (especially if the clay content of the soil is high) and therefore difficult to count the nematodes
- The proportion of nematodes extracted can vary with temperature, causing potential variation in results between samples extracted at different times
- Maximum recovery takes 3–4 days

Equipment
- A basket (or domestic sieve) made with coarse mesh (Fig. 18)
- A dish/tray/plate, slightly larger than the basket
- Tissue paper
- Beakers or containers to wash the extraction into
- Wash bottles
- Waterproof pen
- Knife/scissors
- Weighing scales
- Large bench space.

For soil samples:
- Remove roots from sample and place in a separate dish. Label clearly.
- Using a coarse sieve, remove stones and debris from soil and break up soil lumps.
- In a plastic container (basin, bucket) thoroughly mix the soil sample.
- Remove a measure of soil (e.g. 100 ml).
• Place tissue paper (milk filter, paper napkin etc.) in the plastic sieve/basket (placed on a plastic plate), ensuring that the base of the sieve is fully covered by the tissue. Label.

• Place the soil measure on the tissue in the sieve. It is important that the soil remains on the tissue paper. Spill over results in dirty extractions.

• Add water to the extraction plates. Take care to gently pour water into the plate (dish) and not onto the tissue paper or soil (between the edge of the mesh and the side of the tray).

• Add a set volume to each dish to wet but not cover the soil or root tissue, ensuring there is sufficient not to dry out. More water is needed for soil samples than root material. Add more later if necessary.

• Leave (preferably in the dark) undisturbed for a set period (48 h if possible adding more water if it is likely to dry out. Nematodes from the soil or plant tissue will move through the tissue paper into the water below, resting on the tray/plate.

• After the extraction period, drain excess water from the sieve and the soil into the extraction.

• Remove the sieve and dispose of plant tissue/soil.

• Pour the water from the plate into a labelled beaker (or cup), using a water bottle to rinse the plate. Leave samples to settle.

• For counting the nematodes in the extraction, reduce the volume of water by gently pouring off or siphoning the excess (taking care not to disturb and lose nematodes and sediment), or by passing the extract through a very small aperture sieve (e.g. 20–30 μm). Wash the nematodes off the small aperture sieve into a beaker for counting, or for reserving if sending away or counting later.

**NB:** It is very important to ensure good, consistent labelling of all containers used for each sample, as it is very easy to make mistakes. Root and soil extractions should be labelled separately.

*For root samples:*

Roots can sometimes be divided into separate categories, such as larger tough roots and finer feeder roots. It is useful to extract nematodes separately from each category, as the root tissue texture varies and the type of nematodes invading may also vary,
as well as densities of the same nematode. Extraction efficiencies may also vary, with the nematodes exiting slower from a larger root.

- Gently tap soil off the roots/tubers or rinse under a tap and then gently dab dry with tissue paper. Peel tubers carefully with a knife or kitchen peeler to just below the surface.
- Chop the roots (or tuber peel) finely with a knife or scissors and place in a labelled dish.
- Mix all chopped root material thoroughly
- Remove and weigh a sub-sample (e.g. 5 g) of chopped root material using measuring scales
- Place weighed sub-sample on the tissue paper in the labelled sieve/basket
- Follow the rest of the procedure for soil extraction above

Nematodes will be collected from trays at 24-hour intervals for 2 days and combined into one beaker for each sample. The nematode suspension will then be passed through a 38 µm aperture sieve and nematodes on the sieve back washed into vials and collected in universal bottles for nematode assay.

Killing and fixing nematodes
Nematodes will be concentrated into a small volume of water (30 ml) in a test tube. Triethanolamine, formaldehyde (TAF) and distilled water; normal strength = triethanolamine (2 ml) + 40 % formaldehyde (7 ml) + distilled water (91 ml) will be heated to 99°C and an equal volume quickly added to the nematode suspension. This kills and fixes the nematodes in one process.

Nematode identification
Nematodes from each sample will be counted under a dissecting microscope at low magnification (x40) and then 100 nematodes from each sample identified to genus and species.

Pratylenchus
These species are stout, with body length less than 0.9 mm (Fig. 19). The stylet and lips are highly sclerotised, the lip region is usually not offset from the body and is low and flattened anteriorly giving the appearance of a flat black cap at the head region. The Pharyngeal glands overlap the intestine ventrally. The vulva is posterior and close to the tail and females are monoprodelphic in the interior part of the body. The gubernaculum is simple and does not protrude. Species are most easily differentiated by their tail morphology with *P. goodeyi* having a small mucron or ‘blip’ on the tail tip.

**Figure. 19. Pratylenchus** (worm-like/ vermiform).

*Radopholus similis*

The female nematodes are characterised by a slender body and ranges in length from 0.53 to 0.88 mm. The head is strongly sclerotised internally, composed of 3-4 annules and not offset. The short, stout stylet has well-developed knobs. The pharyngeal glands overlap the intestine dorsally. The vulva is post-equatorial, both branches of the ovaries are fully developed. The tail terminus is almost pointed and striated. The male head is strongly offset and unsclerotized.

*Helicotylenchus multicinctus*

This nematode is best characterised by its C shaped body shape when heat killed. The average life cycle is 30–45 days. An adult female has an oesophagus measuring
22% body length and a stylet of 22.5µ. The lip region is hemispherical, with 3-5 annuli and heavily sclerotized framework. The stylet is well developed and strong. Tail is slightly tapering, with anus marked by a slight depression. Vulva is on the ventral side of the body approximately half way along the body. Males are abundant and shorter and thinner than females.

*Meloidogyne* spp.

In extractions mostly juveniles and sometimes males will be found, as females are sedentary and not mobile, remaining in the root tissue. Newly hatched juveniles have a short free-living stage which invades the root and establishes the feeding site. The juveniles are not longer than 0.5 mm long and have small, poorly developed stylets. Root-knot nematode males are generally rare and also are vermiform and range from 1.1 to 2.0 mm in length. They have distinct lips and strongly developed stylets. In addition, they often have visible spicules, for mating, and a blunt, rounded tail. The female develops at the feeding site through successive moults, swelling into a spherical shape that feeds from cells around the head region. Females are sedentary and do not move from the feeding site. Each female may produce up to 1000 eggs, in a gelatinous sac. There are numerous species of *Meloidogyne* spp. but *M. incognita* and *M. javanica* have mostly been associated with banana.

**Field and greenhouse trials**

*Field trials*

Soil samples to represent different banana cropping sequences will be collected. In each cropping sequence, a trowel or spade will be used to collect soil from five randomly distributed plants from a depth of 5–30 cm. The five sub-samples taken from each single field will then be mixed homogeneously to constitute a composite sample. The soil clods in each composite sample will be carefully broken up with the fingers and sieved gently through a coarse sieve to remove stones and debris. Approximately 500 g of soil will be taken, placed in a plastic bag, labelled, sealed and then kept under cool conditions. The samples will be transported to the laboratory in a cool box and stored at 10°C for nematode assay. Soil samples have to be processed as soon as possible and preferably within 24 h after the collection. Nematodes in the samples will be extracted using the Baermann and wet-sieving techniques.
Scoring of nematode trials

Nematode damage can be evaluated at the same time as field sampling for nematodes. The amount of root damage is estimated visually (as a percentage) using a scoring procedure (Appendix B). The damage score usually has a strong relationship with crop yield losses. Scoring nematode damage provides a rapid indication of the damage at that time.

The number of plants assessed can be one or two, up to 25 or more, depending on the area under assessment. Preferably one person or as few people as possible should undertake the scoring, for consistency of the scoring. The use of score sheets to regularly compare against is advisable for the same reason.

Some judgment may be needed when assessing nematode damage. The score sheets in Appendix B provide examples and a basis upon which to create damage scoring for the crop and nematode damage circumstances. In order to assess lesion damage, functional roots should be taken from the pooled sample, trimmed to ~10 cm length and sliced longitudinally with a knife. Then assess the percentage necrosis (out of 100% root surface) on one half of each of the five roots. Add all the scores together and then divide by 5 for mean percent necrosis damage for each sample (plot). From each sample the number of dead and live roots need to be counted.
Useful reading


Appendix B

Lesion scoring for banana roots

0% 5%

10% 25%

50% 75%

100%